SUBCELLULAR TARGETING: DELIVERING THERAPEUTICS TO THE NEXT LEVEL

by

Mohanad Mossalam

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Pharmaceutics and Pharmaceutical Chemistry

The University of Utah

May 2012

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Mohanad Mossalam**

has been approved by the following supervisory committee members:

Carol S. Lim	, Chair	**12/13/2011** Date Approved
David W. Grainger	, Member	**12/20/2011** Date Approved
James Herron	, Member	**12/13/2011** Date Approved
Philip Moos	, Member	**12/13/2011** Date Approved
Steven Kern	, Member	**12/13/2011** Date Approved

and by **David W. Grainger**, Chair of the Department of **Pharmaceutics and Pharmaceutical Chemistry**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

The targeting of subcellular organelles offers an enhanced therapeutic effect for both small molecules and biologics. The main theme of this research is to use subcellular targeting to elicit or enhance a therapeutic effect, which leads to the hypothesis that *targeting specific organelles in a cell will lead to better drug delivery and improvements in disease therapy*. In this study, we focused on targeting the mitochondria, nucleus, and proteasome. A small molecule, vitamin E, was targeted to the mitochondria by conjugating it with a triphenylphosphonium cation (forming MitoE) taking advantage of the mitochondrial transmembrane potential. MitoE was concentrated in the myocardial mitochondrial of treated mice. It also reduced oxidative stress in hyperglycemic endothelial cells. In addition, the adiponectin protein was used to reduce the oxidative stress in the mitochondria via cAMP/PKA pathway. A new formulation containing adiponectin was developed using a biodegradable triblock copolymer, which forms a hydrogel at body temperature. The goal was to maintain a controlled release of adiponectin from the hydrogel while preserving its antioxidant activity.

The mitochondria were also targeted to deliver the tumor suppressor p53 to induce apoptosis in breast cancer cells via binding to Bcl-XL. p53 was targeted to the outer surface of the mitochondria via fusing to the mitochondrial signal (MTS) from Bcl-XL (XL). It was also inserted into the outer membrane, inner membrane, and matrix by fusing the MTSs from TOM20 (TOM), cytochrome c oxidase (CCO), and ornithine

transcarbamylase (OTC), respectively. The p53-XL construct was the most promising in inducing apoptosis through the p53/Bcl-XL pathway.

In addition to the mitochondria, proteasomal targeting was explored taking advantage of the p53/MDM2 degradation pathway. The designed construct contained a nuclear export signal (NES), a nuclear localization signal (NLS), a ligand binding domain (LBD), and p53. The construct targeted the cytoplasm in the absence of ligand and translocated to the proteasome upon ligand induction. This proteasomal protein switch offers novel treatment therapies for diseases caused by cytoplasmic oncogenic/aberrant proteins.

In summary, this dissertation offers a better understanding on subcellular delivery. The focus on targeting subcellular organelles takes therapeutic delivery to the next level, and may lead to improvements in disease therapy.

TABLE OF CONTENTS

ABSTRACT……...iii

LIST OF FIGURES……...……...viii

LIST OF TABLES……...…….....x

LIST OF ABBREVIATIONS……..……...xi

ACKNOWLEDGMENTS……...…….....xiv

Chapter

1. BACKGROUND AND SIGNIFICANCE……..1

Summary……..…...1
Background…….2
Reducing Oxidative Stress in Cardiovascular Model……..……...2
Oxidative Stress in the Hearts of Diabetic Patients…….....….....6
Adiponectin……...…….....9
Antioxidants……..……..…….....11
Diabetic Mitochondria……..……...…….13
Local Controlled Delivery of Drugs Using Biodegradable Polymer..……..15
Inducing Apoptosis in Breast Cancer……..……..17
The p53 Protein…….……...…….....19
Mitochondria in Cancer Cells…….....…….....26
Targeting the Nucleus……..……...…….....29
Proteasomal Targeting……..……...…….32
Gene Therapy……..……..33
Statement of Objectives…….....34
References…….....37

2. CONTROLLING SUBCELLULAR DELIVERY
TO OPTIMIZE THERAPEUTIC EFFECT …….63

Abstract…….63
Introduction……...64
Cytosolic Delivery……66
The Endosome/Lysosome…….....73

Nucleus……75
Targeting the Nucleolus…….....81
Mitochondria…….....84
Endoplasmic Reticulum……90
The Golgi Apparatus…….....92
Peroxisomes……..96
Proteasome..……100
Future Perspective…….....101
Executive Summary…….....102
Acknowledgments……104
References……105

3. REVERSAL OF OXIDATIVE STRESS IN ENDOTHELIAL CELLS BY CONTROLLED RELEASE OF ADIPONECTIN…….....139

Abstract……139
Introduction……..140
Materials and Methods…….....141
Results and Discussion……....143
Conclusions……..151
Acknowledgments……151
References……152

4. SOLID PHASE SYNTHESIS OF MITOCHONDRIA TRIPHENYLPHOSPHONIUM-VITAMIN E USING A LYSINE LINKER FOR REVERSAL OF OXIDATIVE STRESS……...155

Abstract…….....155
Introduction…….....156
Materials and Methods…….....158
Results and Discussion…….....162
References…….....170

5. INDUCTION OF APOPTOSIS THROUGH COMPARTMENT-SPECIFIC MITOCHONDRIAL TARGETING OF P53…….....175

Abstract…….....175
Introduction……..176
Materials and Methods…….179
Results……187
Discussion……203
Acknowledgments…….....210
References…….....211

6. DESIGNING A PROTEIN SWITCH TO CONTROL PROTEASOMAL TARGETING…….....217

Abstract…….....217
Introduction……..217
Materials and Methods…….....221
Results and Discussion…….....223
Conclusion……...230
References…….....233

7. CONCLUSIONS AND FUTURE WORK…….....237

Conclusions…….....237
Adiponectin Released from the Triblock Copolymer Maintained Its Antioxidant Effect……...237
A Faster Synthesis of Mitochondrially Targeted Vitamin E for Reversal of Oxidative Stress……238
Mitochondrial p53 Activity Depends on the Mitochondrial Signal Used…….239
Proteasomal Localization Controllable Construct……...240
Future Work……241
Antioxidant Potential of Controlled Released Adiponectin *In Vivo*……...242
Conjugation of Antioxidants to TPP Using Solid Phase Synthesis ……243
Targeting Small Domains of p53 to Mitochondrial Bcl-XL…….……...244
Creating "Super" Mitochondrial p53……...245
Enhanced Dimerization of p53 to Target Mitochondrial Bak…….....246
Substituting Tetramerization Domain of p53 with Bcr Coiled Coil……247
Fusing 4 NLSs to p53……..248
Controlled Nuclear Targeting of p53-CC……249
Application of the Protein Switch: Depleting Cytoplasmic Bcr-Abl in Chronic Myelogenous Leukemia……...249
Validating Out Therapy *In Vivo* Using an Adenovirus Vector……250
References……252

LIST OF FIGURES

Figure		Page
1.1	A summary of the disease targets, drugs used, and expected outcome	3
1.2	A cell overview of the subcellular targets of the different therapeutics in this thesis	4
1.3	Electron transfer chain in mitochondria	7
1.4	Increased mitochondrial targeting of small molecules	14
1.5	Full-length p53	21
1.6	The extrinsic and intrinsic apoptosis pathways	23
1.7	The Bcl2 family proteins	28
2.1	Drug targeting to cellular organelles	65
2.2	EGFP fused to different targeting signals	72
2.3	Protein switch mechanism	82
3.1	Glucose effect on ROS production	144
3.2	The gAdp dose-response in reducing ROS	145
3.3	*In vitro* release study	147
3.4	SDS PAGE for the released adiponectin	148
3.5	Effect of the released gAdp on ROS	149
3.6	Effect of the released gAdp cAMP	150
4.1	The structure of MitoE	164

4.2 MALDI-TOF mass spectrometry of MitoE…….....165

4.3 LC/MS data…….....167

4.4 Effect of Vitamin E and MitoE on reducing ROS……...168

5.1 The mitochondrial apoptotic pathway of p53……..178

5.2 Colocalization of MTS constructs and MitoTracker Red…….....188-189

5.3 Luciferase assay……....191-192

5.4 The activation of caspase-9 …….....194

5.5 TUNEL assay……...195

5.6 Annexin-V assay……..197

5.7 7-AAD assay…….....198-199

5.8 Rescue experiments……..201-202

5.9 Speculated MTS-p53 apoptotic mechanism……....207

6.1 The enzymes and reactions of the ubiquitin-proteasome system…….219

6.2 Change in protein localization on ligand induction…….224

6.3 The proteasomal switch mechanism……...…….226

6.4 Representative time dependent targeting for protein constructs. ……....227

6.5 Change in EGFP-PS-p53 ΔMBD localization on ligand induction……228

6.6 The effect of nutlin-3……...229

6.7 The effect of LMB……...231

LIST OF TABLES

Figure		Page
2.1	Cytosolic targeting of drug therapeutics……...69-70	
2.2	Methods to link NLSs to DNA-based therapeutic agent…….....78	
2.3	Mitochondria targeting signals/motifs and delivered therapeutic agents.....88	
2.4	Endoplasmic reticulum targeting signals/motifs and delivered therapeutic agents…….....93	
2.5	Peroxisomal targeting signals and therapeutics delivered…….....100	
5.1	A list of mitochondrial targeting signals……..180	
5.2	A summary of collected data and speculated mechanism…….....209	

LIST OF ABBREVIATIONS

Adp	Adiponectin
AdR1	Adiponectin receptor-1
AdR2	Adiponectin receptor-2
AMPK	5’ Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cells
BH	Bcl2 homology
cAMP	Cyclic adenosine monophosphate
CCO	Mitochondrial signal from cytochrome c oxidase
CKII	Casein kinase II
CML	Chronic myelogenous leukemia
CPP	Cell penetrating peptides
CRM1	Chromosome maintenance region 1
DBD	DNA binding domain
Dex	Dexamethasone
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	Dimethylformamide
DRB	5,6-dichloro-1-ribo-furanosylbenzimidazole
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-ligating enzyme
E3	Ubiquitin ligase
EGFP	Enhanced green fluorescent protein
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
$FADH_2$	Flavin adenine dinucleotide
fAdp	Full-length adiponectin
Fmoc	Fluorenylmethoxycarbonyl
gAdp	Globular form of adiponectin
GR	Glucocorticoid receptor
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HECT	Homologous to the E6-AP carboxy terminus
HER-2	Human epidermal growth factor receptor 2
HMW	High-molecular weight
HOBt	HOBt-6-carboxamidomethyl polystyrene
Hsp70	Heat shock cognate protein 70
IBB	Importin β binding
IRD	Infantile Refsum’s disease

LBD	Ligand binding domain
LC/MS	Liquid chromatograph/mass spectrometry
LMB	Leptomycin B
Lys	Lysine
MBD	MDM2 binding domain
MCF-7	Human breast adenocarcinoma cell line
MDM2	Mouse double minute 2
MitoE	Mitochondrial Vitamin E
MitoQ	Mitochondria ubiquinol
MnTBAP	Mn (III) tetrakis (4-benzoic acid) porphyrin chloride
MPP	Mitochondria processing peptidase
mPTS	Membrane protein targeting signal
mtDNA	Mitochondria DNA
MTS	Mitochondrial targeting signal
Mtt	Methyltrityl
NADH	Nicotinamide adenine dinucleotide
NALD	Neonatal adrenoleukodystrophy
NF-κB	Nuclear factor-κB
NES	Nuclear export signal
NLS	Nuclear localization signal
NoLS	Nucleolus localization signal
NPC	Nuclear pore complex
ob/ob	Obese/Diabetic mice
OTC	Mitochondrial signal from ornithine transcarbamylase
Parc	p53-associated Parkin-like cytoplasmic protein
PBD	Peroxisomal biogenesis disorder
PCC	Pearson's correlation coefficient
PE	Phosphatidylethanolamine
PEG	Poly (ethylene glycol)
PEI	Polyethyleneimine
PKA	Protein kinase A
PLGA	Poly (lactide-co-glycolide)
PMP	Peroxisomal membrane proteins
PNA	Peptide nucleic acid
PP2A	Protein phosphatase 2A
PPARγ	Peroxisome proliferator-activated receptor gamma
PRD	Proline rich domain
PS	Protein switch
PTS	Peroxisomal targeting signal
RES	Reticuloendothelial system
RING	Really interesting new gene
ROS	Reactive oxygen species
SLTB	Shiga-like toxin subunit B
snRNP	Small ribonucleprotein particles
SOD	Superoxide dismutase
SRP	Signal recognition particle

STB	Shiga toxin subunit B
T47D	Human ductal breast epithelial tumor cell line
TCA	Tricarboxylic acid cycle
TD	Tetramerization domain
TIM	Translocase of the inner membrane
TPP	Triphenylphosphonium
TNF-α	Tumor necrosis factor-α
TOM	Translocase of the outer membrane
TOM	Mitochondrial signal from TOM20
VZV gpI	Varicella-zoster virus glycoprotein I
WGA	Wheat germ agglutinin
XL	Mitochondrial signal from Bcl-XL
XO	Xanthine oxidase

ACKNOWLEDGEMENTS

Foremost, I would like to thank my beautiful wife, Nichole, for her wholehearted support during my graduate school years. We married in my first year of graduate school and she was always there for me even at times when I was too stressed out to show my appreciation. I would not be where I am today if it were not for the sacrifices she made. I am also thankful for my son, Nourudeen. He never stops putting a smile on my face every time I walk in the door. I hope one day he reads this dissertation to know that I am doing all this for him. Who knows, maybe he will actually continue this work someday.

I would also like to thank my dad for his encouragement and sacrifices he made so I could finish my education at the University of Utah. I will always be indebted to him for he is the reason for what I have become. I am also grateful for my siblings. I am thankful for my sister Maha, who burdened herself with so much to help me at times when I needed help. My older brother, Mohamed, has always been there for me with his wisdom and kindness. And my sister, Mona, will always be the joy of the family and the center of attention. I would also like to acknowledge all my friends, who are also my family. If I were to mention everyone by name, it would take a few pages. I am thankful for all of them who stood behind me every step of the way.

I am very grateful for having Dr. Carol Lim as my mentor. At a time when all doors were closed and all hope was gone, Dr. Lim embraced me in her lab. This when my life was changed forever. Dr. Lim is an outstanding mentor. She finds the best in you and

because of that you become your best. She has created a lab environment where everyone collaborates and an environment where people excel out of respect for her and not fear. I would not have achieved anything in my graduate career if it were not for her encouragement. What she has instilled in me will always have an impact on my future success.

I would also like to thank everyone in the Lim lab for their help and support in the lab and outside of the lab. I would like to thank Andy Dixon, Jonathan Constance, Rian Davis, David Woessner, Ben Bruno, Geoff Miller, and Shams Reaz. A special thanks goes to Karina Matissek and Abood Okal for working with me as a team on the p53 project. I would not be graduating if it were not for the experiments and scientific discussions we had together. I have built strong friendship with everyone in this lab that I will cherish forever. I would like to acknowledge all the students, who worked directly under my mentorship specially Julien Ta and Katharina Opper.

I would like to thank my supervisory committee members, Dr. David Grainger, Dr. James Herron, Dr. Philip Moos, and Dr. Steven Kern, for their scientific advice and input. On a personal level, I appreciate all what Dr. Grainger has done for me. I have spent numerous hours in his office soliciting help and support. And despite his busy schedule, he never turned me down. He was always there to offer his wisdom and input.

This work is in dedication to my mother, who passed away in 2000 from cancer. She was the only one I went to for emotional support. Her life and her passing away is what drove this work. I would also like to dedicate this work to my mother-in-law for her battle and subsequent remission from breast cancer. I am thankful to GOD for having all these people in my life.

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Summary

Subcellular targeting has been a challenge for delivering therapeutics whether they are small molecules or biologics. Drugs and proteins have different effects depending on their subcellular localization. For instance, Bcr-Abl acts as an oncogenic protein in chronic myelogenous leukemia (CML) when in the cytoplasm while it induces apoptosis when delivered to the nucleus (*1*). Incorrect localization of proteins destined for different organelles can lead to disease (*2*). The main theme of this research is to use subcellular targeting to elicit or enhance a therapeutic effect, which leads to the hypothesis that *targeting specific organelles in a cell will lead to better drug delivery and improvements in disease therapy*.

This introductory chapter will provide an overview of the thesis in brief followed by Chapter 2, a review on therapeutic delivery of drugs to subcellular compartments (mitochondria, nucleus, proteasome, endoplasmic reticulum, peroxisome, Golgi apparatus, and nucleolus). Targeting a specific organelle can be achieved by chemical or biological means. The next level of therapeutic delivery will include specific targeting to organelles by imitating nature with an efficiency required to mitigate specific pathologies. Some of the methods reviewed to target specific organelles were further investigated by delivering

therapeutics to the subcellular compartments. Two disease targets are investigated: cardiovascular disease and breast cancer (Figure 1.1). The next four chapters discuss delivery of a drug agent to a particular subcellular compartment for disease treatment (Figure 1.2). Chapter 3 focuses on delivery of a therapeutic protein (adiponectin) to reduce reactive oxygen species (ROS) in the mitochondria for treatment of diabetes; Chapter 4 focuses on the delivery of a small molecule antioxidant to the mitochondria for treatment of cardiovascular disease; Chapter 5 describes delivery of p53 to the mitochondria for cancer therapy, and Chapter 6 discusses nuclear/proteasomal targeting of p53 directly and with a protein switch developed in our laboratory (*3, 4*). Finally, Chapter 7 provides conclusions and future directions.

The introduction chapter follows the organization in the chart in Figure 1.1. Part A discusses the reduction of the oxidative stress in cardiovascular disease including an introduction to (A1) oxidative stress in the hearts of diabetic patients, (A2) adiponectin, (A3) antioxidants, (A4) diabetic mitochondria, and (A5) biodegradable polymers for drug delivery. Part B discusses inducing apoptosis in breast cancer including a background on (B1) p53 protein, (B2) mitochondria in cancer cells, (B3) nuclear targeting, (B4) proteasomal targeting, and (B5) gene therapy.

Background

Reducing Oxidative Stress in Cardiovascular Models

Cardiovascular diseases encompassing disorders of the heart and blood vessels remain the leading cause of death in diabetic patients. These diseases include coronary

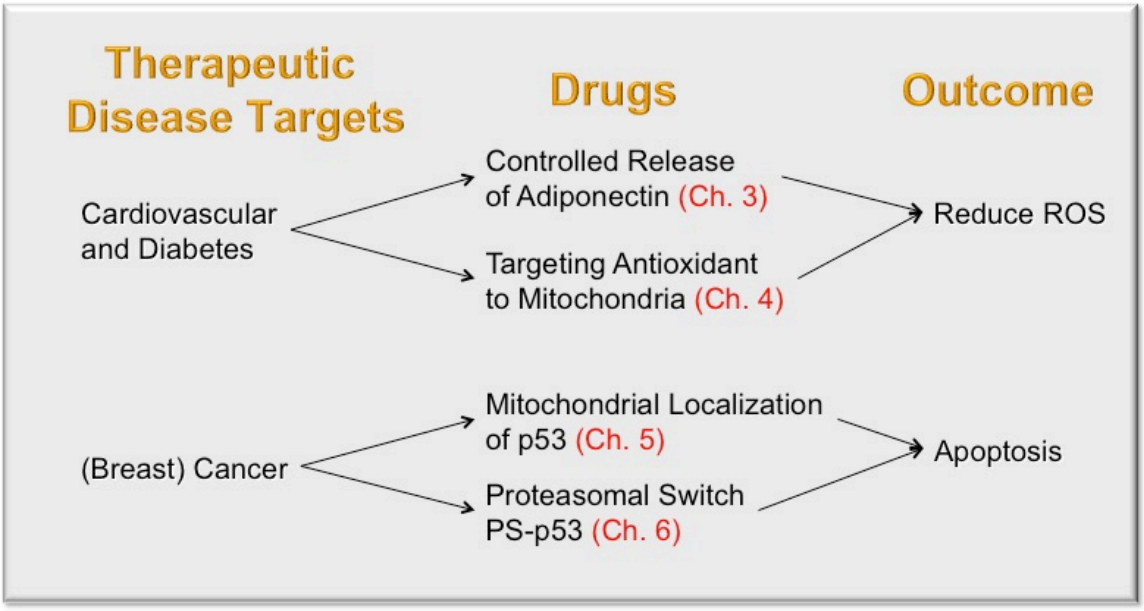


Figure 1.1. A summary of the disease targets, drugs used, and expected outcomes.

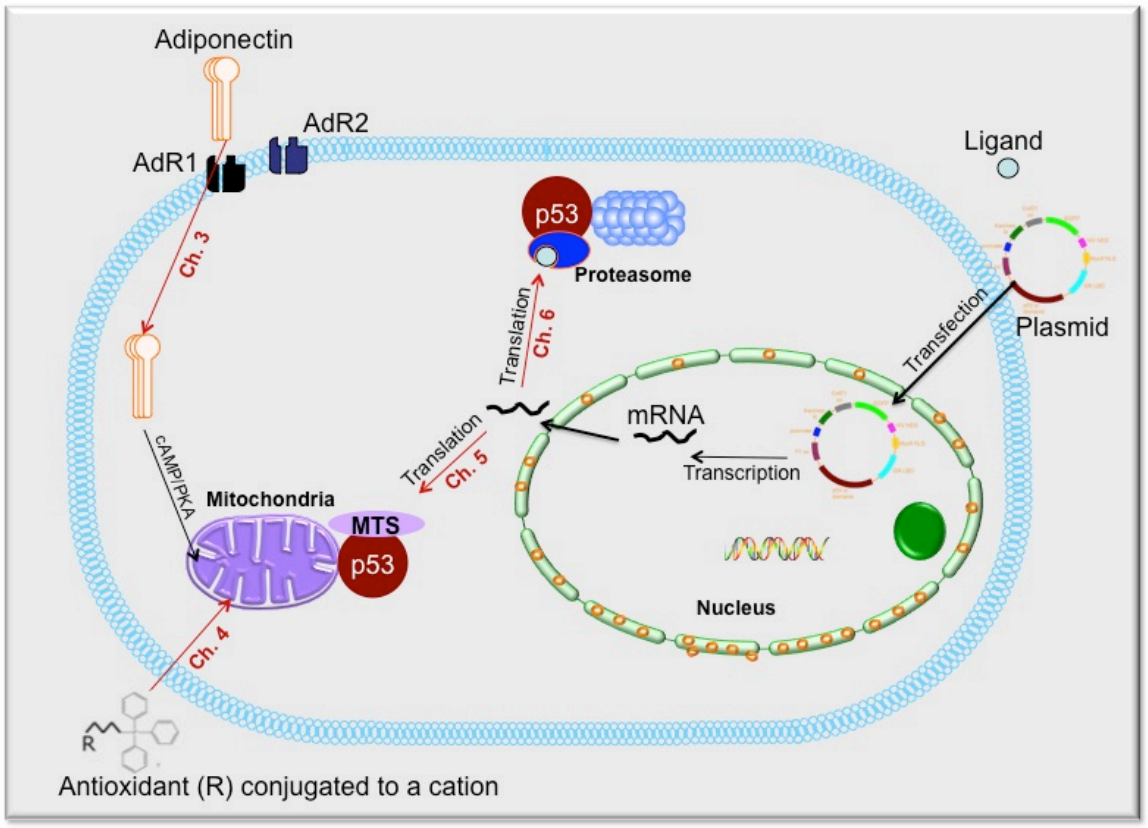


Figure 1.2. A cell overview of the subcellular targets of the different therapeutics in this thesis. Chapter 2 covers the various approaches in targeting the different organelles. Chapter 3 focuses on the delivery of adiponectin protein through its receptors (AdR1 and AdR2) to reduce ROS in the mitochondria via a cAMP-dependent protein kinase (PKA) pathway. Chapter 4 discusses the delivery of small molecules such as vitamin E (R) to the mitochondria to reduce the oxidative stress by conjugation to a cation (triphenylphosphonium). Chapter 5 investigates the gene therapy of p53 fused to mitochondrial targeting signals (MTS) for cancer therapy. Chapter 6 examines the proteasomal targeting of p53 protein switch upon ligand addition.

heart disease, hypertension, cerebrovascular disease, heart failure, peripheral vascular disease, congenital heart disease, and rheumatic heart disease (*5*). Subcellular targeting is crucial in reducing oxidative stress in diabetic patients, who have a high risk for atherosclerosis and congestive heart failure (*6*). Increases in fatty acid flux augment the formation of reactive oxidative species (ROS) in the mitochondria causing a reduction in adenosine triphosphate (ATP) synthesis by activating uncoupling proteins (*7*). Uncoupled mitochondria contribute to lowering myocardial energy, which causes myocardial dysfunction (*8*). The vascular function is ROS modulated either by direct oxidative damage or by activating cellular signaling pathways (*9*). ROS can also modulate vascular function through endothelial dysfunction, decreased nitric oxide (NO) bioavailability, impaired contractility, and platelet aggregation (*10-13*). In addition, oxidative stress diminishes insulin signaling through cellular serine/threonine pathways (*14*). More specifically, the insulin receptor substrate-1 gets phosphorylated under oxidative stress, which could be improved by antioxidant agents (*14-16*). Moreover, some ROS serve as important signaling molecules that regulate several physiological mechanisms, such as vascular tone, growth, and remodeling (*10*). In the cell, there is a delicate balance between ROS production and destruction (*17*).

Decreasing the oxidative stress in the mitochondria has been a therapeutic target to increase myocardial activity. Antioxidants have been used to decrease ROS levels in cells (*18, 19*). However, an additional approach to relieving oxidative stress within mitochondria is to augment subcellular concentrations of antioxidants by selectively targeting them to the mitochondria (*20-22*). In addition to antioxidants, proteins such as

adiponectin, which is an anti-diabetic protein, have an impact on reducing oxidative stress (*23*).

Indeed, antioxidants and adiponectin could be used therapeutically to reduce oxidative stress in the hearts of diabetic patients. To avoid using high doses of such therapeutic agents, sustained delivery of the substances locally to the heart can be achieved by directly injecting the myocardium with biodegradable triblock copolymer containing the drug. The sustained delivery could also be achieved when the polymer formulation is delivered subcutaneously (*24-26*). The biodegradable triblock copolymer allows the delivery of insoluble, unstable or expensive substances at high local concentrations. This is the topic of Chapter 3 of this thesis. In addition, antioxidants could be conjugated to cations to increase their mitochondrial targeting, where ROS is being produced and metabolized (Chapter 4).

Oxidative Stress in the Hearts of Diabetic Patients

Diabetic patients have a higher risk of dying of a heart attack or a stroke than non-diabetic patients. There is an increase in fatty acid flux in patients with diabetes, which increases the delivery of reducing equivalents to the respiratory chain. In mitochondria, the electron transport chain passes electrons from the reducing equivalents (flavin adenine dinucleotide, $FADH_2$, and nicotinamide adenine dinucleotide, NADH) to oxygen (Figure 1.3). This results in the pumping of protons from the matrix to intermembrane space via NADH dehydrogenase, cytochrome c reductase, and cytochrome c oxidase. As a result, an electrochemical gradient is established, which activates ATP synthase to pump protons back to the matrix and activates ADP to ATP. In the mitochondrial

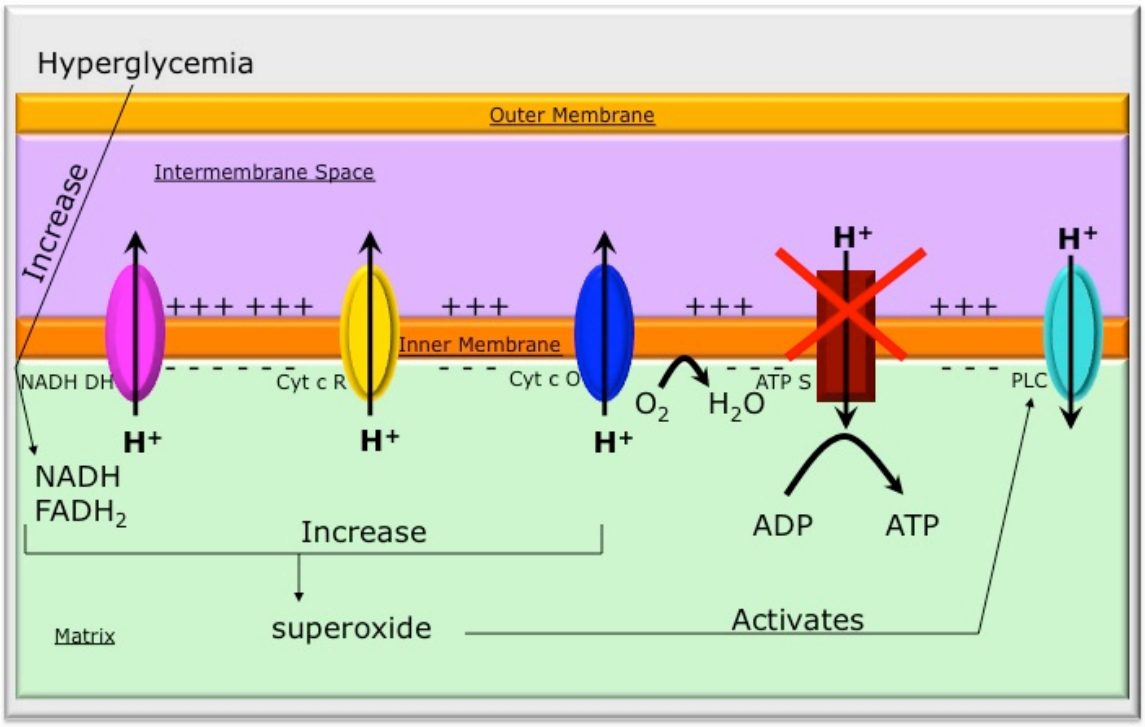


Figure 1.3. Electron transfer chain in mitochondria affected by increase in ROS due to hyperglycemia. Hyperglycemia increases the delivery of reducing equivalents to the respiratory chain. In mitochondria, the electron transport chain passes electrons from the reducing equivalents ($FADH_2$ and NADH) to oxygen. The protons are then pumped from the matrix to intermembrane space via NADH dehydrogenase (NADH DH), cytochrome c reductase (Cyt c R), and cytochrome c oxidase (Cyt c O). The resulting electrochemical gradient activates ATP synthase (ATP S) to pump protons back to the matrix and forms ATP. This process also forms superoxide radicals, which activate the proton leak uncoupling protein (PLC). PLC pumps protons back into the matrix without forming ATP.

respiratory chain, oxygen is partially reduced to form superoxide radicals. ROS activate the proton leak uncoupling proteins, which pump protons back into the matrix without forming ATP (Figure 1.3). ROS are also generated via TCA cycle enzymes (*27*), NADPH oxidase (*28*), xanthine dehydrogenase/oxidase (*29*), mitochondrial monoamine oxidase (*29*), superoxide dismutase (*30*), peroxisomal acyl-CoA oxidases (*31*), and myeloperoxidases (*32*). ROS are also formed from endogenous enzymatic and non-enzymatic reactions or from external stimuli (UV and high-energy irradiation) and chemicals (air pollutants) (*29*). Superoxides are also converted to other ROS such as hydroxyl radicals, which can cause damage to cells and tissues. The cell produces a range of superoxides such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), hypochlorous acid (HOCl), superoxide anion radical ($O_2^{\cdot -}$), and singlet oxygen ($^1O_2$) (*29*).

Increase in ROS production is associated with diabetes, hypercholesterolemia, aging, hypertension, and mechanical injury (*10*). There are different mechanistic pathways to increase ROS production. NADPH oxidase enzymes (Nox enzymes) are a major source of ROS in endothelial cells. The activation of Nox involves activation of the tyrosine kinase c-Src (*33*). Nox enzymes also activate xanthine oxidase (XO), which uses oxygen as an electron acceptor from NAD^+ to form oxygen radicals and hydrogen peroxide (*34, 35*). Another mechanism of ROS generation is uncoupled endothelial nitric oxide synthase (eNOS). eNOS usually reduces nitrite anion (NO_2^-) to nitric oxide (NO), which acts as a protective compound to the heart and obstructs the mitochondrial electron transfer (*36, 37*). However, the uncoupling of eNOS or in the absence of L-arginine, eNOS stops transferring electrons to L-arginine and starts transferring electrons to oxygen molecules forming oxygen radicals (*38, 39*). Nitric oxide is also produced via the

activation of the fuel sensor, AMP-activated protein kinase (AMPK), which is responsible for the ameliorative effect on mitochondrial function and biogenesis (*40, 41*). This work provides a possible solution to reduce myocardial oxidative stress by exploring the protein kinase pathway (*42*) as well as targeting the mitochondria with antioxidants (*43*).

Adiponectin

In human subjects, studies have shown the influence of adipose tissue on obesity, insulin resistance, and cardiovascular disease. Adipose tissue functions as an endocrine organ by secreting leptin, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-10, plasminogen activator inhibitor type 1, retinol binding protein-4, and adiponectin (*44, 45*). All secreted proteins are directly involved in disorders linked to diabetes. It has been shown that adiponectin levels are reduced in patients with obesity and cardiovascular disease (*46, 47*). Clinical studies have linked low adiponectin concentration to insulin resistance (*48-51*), hypertension (*52*), dyslipidemia (*53*), and coronary artery disease (*54-57*).

Adiponectin is present in the plasma, ranging between 3 to 30 μg/ml (*46, 58*). There is a negative correlation between adiponectin levels and body mass index, which means its secretion is decreased with accumulated fat (*46*). Adiponectin expression is induced by the nuclear receptor, PPARγ (peroxisome proliferator-activated receptor gamma), which binds to the PPARγ response element in the promoter region of the adiponectin gene (*59*). Interestingly γ- and α-tocopherol enhance the expression of

PPARγ (*60-63*). Treatment with α-tocopherol shows elevated levels of adiponectin (protein and mRNA) in both adipose tissue and circulation (*64, 65*).

Adiponectin receptors (AdipoR1 and AdipoR2) are found in liver and muscle tissue, respectively (*66*). Adiponectin increases fatty acid oxidation in skeletal muscle and reduces glucose production in the liver (*67*). Adiponectin improves hyperglycemia when bound to its receptors (*68*). This binding increases the intracellular calcium levels, which activates the protein kinases necessary for the processing of glucose (*69*). Eventually, this process enhances skeletal muscle glucose uptake.

Adiponectin exists in trimer, hexamer, and high-molecular weight (HMW) forms (*70, 71*). The protein contains a collagen-repeat domain at the amino terminus and a globular domain at the carboxy terminus. Because it is thought that the globular domain (gAdp) is the active form of adiponectin, gAdp has been more widely used than the full-length form (fAdp). Adiponectin plays an important role in angiogenesis (*72, 73*), endothelial function (*74*), glucose regulation (*75, 76*), and fatty acid catabolism (*75, 77*). The protein improves insulin control (*78*), blood glucose (*79*), and triglyceride levels (*80*). It has been reported that adiponectin increases nitric oxide production through AMP-activated protein kinase (AMPK) and phosphorylation of eNOS (*81, 82*). Adiponectin also blocks the generation of ROS (*38*), which could be one of the major factors why patients with obesity and diabetes, who have low concentrations of adiponectin, develop ROS overproduction. Chapter 3 will further explore the activity and delivery of adiponectin in endothelial cells.

Antioxidants

The production of oxidative stress and reduced antioxidant reserve are related to congestive heart failure **(*83*)**. Cellular ROS is countered by endogenous antioxidants such as vitamin C, vitamin E, superoxide dismutase (SOD), and glutathione peroxidase **(*84*)**. Halliwell (*85*) defines an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate." The cell contains oxidizable substrates such as proteins, lipids, carbohydrates, and DNA. Antioxidants either detoxify the free radicals by scavenging them or prevent their formation by eradicating their precursors (*29*). Antioxidants could be divided into different categories: vitamins (e.g., α-tocopherol, ascorbic acid, β-carotene), inorganic compounds (e.g., selenium), synthetic compounds (e.g., butylated hydroxyanisole), and plant derived polyphenols (*29*).

Superoxide dismutase (SOD) is an example of a naturally occurring antioxidant that scavenges ROS and converts them to less reactive species. Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) is a cell permeable catalytic SOD mimetic and peroxynitrite scavenger. MnTBAP has been shown to reduce mitochondrial ROS *in vitro* (*86, 87*). It was also reported that MnTBAP reduces mitochondrial ROS overproduction and improves markers of oxidative injury in the liver of obese (ob/ob) mice (*88*). On the other hand, vitamin C, a water-soluble antioxidant, scavenges ROS and free radicals and protects low-density lipoprotein against oxidation, reduces oxidized glutathione, and functions as a co-antioxidant to vitamin E (*89, 90*). Vitamin C also enhances nitric oxide production and endothelial cell proliferation (*91, 92*). Some studies have shown that adequate intake of vitamin C can lower the risk of heart disease (*93, 94*).

Another naturally occurring antioxidant, further discussed in Chapter 4, is vitamin E, a fat-soluble antioxidant with eight naturally occurring vitamer forms. The most common forms are the γ- and α-tocopherols. The latter has also shown activity in expressing certain genes involved in cell cycle regulation, inflammation, cell signaling, and lipid uptake (*95*). Vitamin E has a positive effect on insulin sensitivity and the prevention of type-2 diabetes (*96-98*) due to its antioxidant capacity (*99, 100*). Alpha-tocopherol is a chain-breaking antioxidant, where it interrupts the formation of lipid-derived oxygen- and carbon-centered free radicals (*29*). These radicals are formed via a chain reaction: initiation, propagation, and termination. Alpha-tocopherol has also shown activity in expressing certain genes involved in cell cycle regulation, inflammation, cell signaling, and lipid uptake (*95*).

It has been reported that naturally occurring antioxidants such as vitamin C and vitamin E are reduced in diabetics (*101, 102*). However, supplementing these vitamins in diabetic populations has been ineffective or inconclusive. In general, clinical trials with antioxidants have failed to show any significant clinical benefit. There is a possibility that this failure is due to the lack of antioxidant accumulation in the mitochondria, the proper target for scavenging ROS (*29*). Meanwhile, administering high doses of antioxidants such as vitamin E may be harmful or act as pro-oxidant (*103*). Chapter 4 will provide an alternative pathway to reduce ROS by antioxidant targeting of the mitochondria. Using modified antioxidants to specifically target the mitochondria should allow use of smaller doses of antioxidants, reducing toxicity and pro-oxidant effects.

Diabetic Mitochondria

The mitochondria play roles in cellular energy metabolism, apoptosis, Ca^{2+} homeostasis, and cell signaling (*104-107*). Because of these roles, the mitochondria have been under investigation for therapeutic targeting (*19-21, 108*). Mitochondrial dysfunction plays a central role in the development and progression of tumors (*109-111*). In addition to its production of ATP, the mitochondria have several functions that are tissue specific (*112*). Myocardial mitochondria are involved in the generation of energy, the regulation of apoptosis, and the generation and detoxification of ROS. Mitochondrial dysfunction initiates apoptosis and necrosis resulting in organ failure. Disrupting the mitochondrial Ca^{2+}, ATP, or ROS metabolism plays a role in different diseases such as diabetes, obesity, heart failure, stroke, aging, cancer, and neurodegenerative diseases (*113*). The mitochondria utilize 90% of consumed oxygen for ATP synthesis and oxidative phosphorylation. This process in the electron transfer chain is responsible for ROS production (Figure 1.3). The cell has its own antioxidant defenses such as glutathione, catalase, and superoxide dismutase to prevent oxidative stress (*114-118*). Maintaining this ROS/antioxidant ratio is imperative for cell signaling (*29*). Any imbalance between ROS generation and destruction is associated with chronic disease (*119*). Since the mitochondria produces and metabolizes ROS, targeting antioxidants to the mitochondria has been a focus of interest (*120*). This is achieved by conjugating an antioxidant to a lipophilic cation. The positive charge enables mitochondrial accumulation 100-1000 times higher due to the high inner mitochondrial membrane potential (Figure 1.4) (*121, 122*).

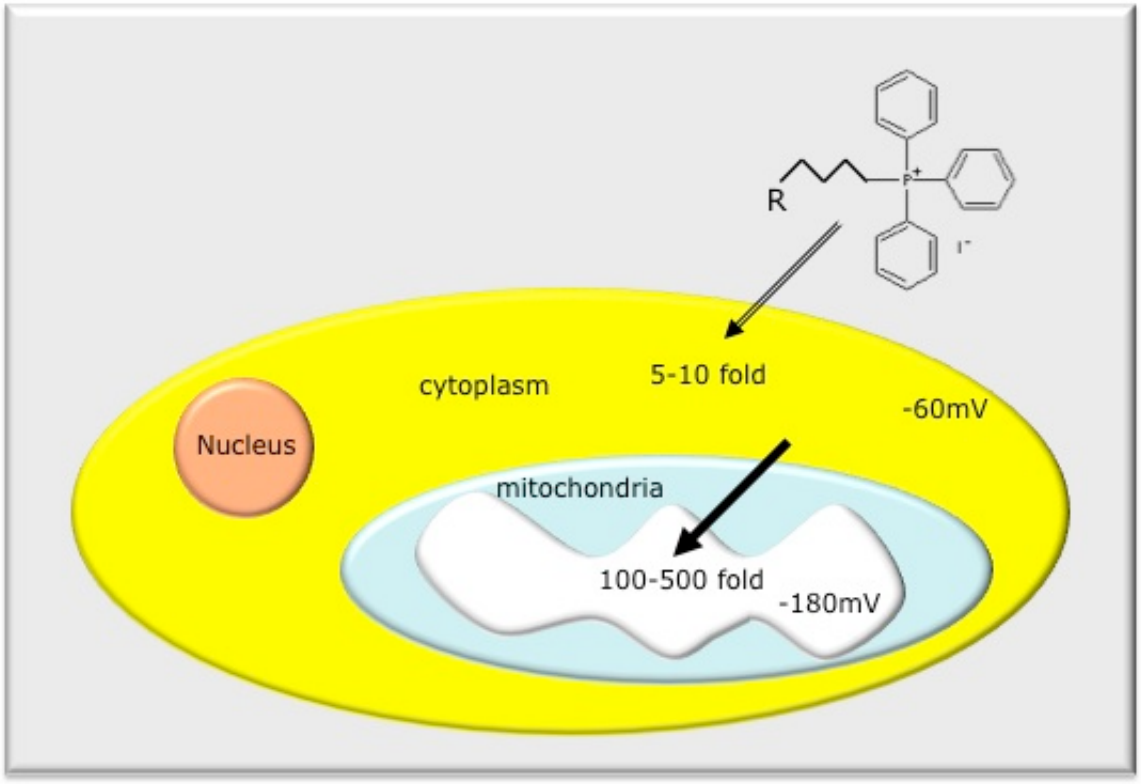


Figure 1.4. Increased mitochondrial targeting of small molecules. The delivery of small molecules such as vitamin E (R) to the mitochondria to reduce the oxidative stress by conjugation to a cation (triphenylphosphonium). This conjugation increases the mitochondrial targeting by 100-500 fold due to the -150 to -180 mV mitochondrial transmembrane potential.

Mitochondria contain outer and inner membranes composed of phospholipid bilayers and proteins. This results in five distinct compartments: the outer membrane, the intermembrane space, the inner membrane, the cristae space and the matrix. Small molecules can permeate mitochondrial outer membrane but not the inner membrane (*123*). There are two ways for mitochondrial targeting of large molecules and small molecules especially to the matrix: one based on the high negative potential in the matrix, and another based on import through translocases in the outer and inner membranes. Small molecules can diffuse through pores formed by the spanning β-barrel protein in the mitochondrial outer membrane. Electrons are transferred to O_2 in the mitochondrial respiratory chain. This leads to a proton gradient that drives the production of ATP via the ATP synthase. Consequently, a -150 to -180 mV mitochondrial transmembrane potential is generated. Lipophilic cations, such as rhodamine, and triphenylphosphonium (TPP), increase accumulation inside mitochondria due to the high negative charge in the mitochondrial matrix (Figure 1.4) (*122, 124, 125*). They initially accumulate 5-10 fold in the cytoplasm due to the -30 to -60 mV plasma membrane potential. Then they permeate the mitochondrial lipid bilayers and accumulate 100-500 fold in the matrix due to the -150 to -180 mV mitochondrial transmembrane potential (*126*).

A. 5. Local Controlled Delivery of Drugs Using Biodegradable Polymer

Drugs including antioxidants, modified antioxidants, and anti-diabetic proteins, can be administered directly to the myocardium or subcutaneously by using an injectable biodegradable triblock copolymer delivery system without surgery (*24, 127, 128*). The biodegradable triblock copolymer allows the delivery of insoluble, unstable or expensive

substances at high local concentrations, due to the combination of hydrophilic poly (ethylene glycol) (PEG) or the B-block and hydrophobic poly (lactide-co-glycolide) (PLGA) or the A-block (*26*). The triblock is either arranged as BAB or ABA. The polymer takes advantage of the hydrophilic and hydrophobic blocks. The hydrophobic interactions are increased at high temperatures forming physical crosslinks resulting in the formation of the gel state (*129*). The hydrophilic block is responsible for the degradation rate and matrix permeability. The gelation temperature is controlled by the PEG/PLGA ratio, glycolide/lactide ratio, and the molecular weight. In addition, this formulation does not affect the activity of the loaded proteins as in the case of PLGA microspheres. Proteins lose activity when exposed to organic solvent/water interface during microsphere preparation (*130, 131*).

Administering drugs using biodegradable triblock copolymer allows sustained drug release (several weeks) of therapeutic substances subcutaneously or directly to the heart without the potential of deleterious systemic effects (*132-134*). The polymer is also biocompatible and biodegradable, designed to be unreactive with the surrounding environment and prevents the need for retrieval of the implant. Furthermore, the polymer biodegrades, releasing the drug in a controlled manner then disappears from the injection site in a timely fashion. The polymer is in the liquid form at low temperatures and spontaneously gels at body temperature (37°C) (*25, 135*). Local controlled delivery will reduce the total dose of antioxidant and hence prevent the possibility of pro-oxidant activity that may occur with administering high doses. This approach was tested on delivering adiponectin, further discussed in Chapter 3.

Inducing Apoptosis in Breast Cancer

Mitochondrial targeting can also be utilized for gene therapy of breast cancer. Breast cancer starts in the tissue of the breast either in the lobules (lobular carcinoma) or the ducts (ductal carcinoma). When the cancer spreads into other breast tissues, it is defined as invasive. In addition, if the breast cancer is sensitive to the hormone estrogen, causing it to grow, it is defined as estrogen receptor-positive cancer and marked with estrogen receptors on the surface of their cells. Despite the fact that breast cancer mortality is falling in the Western world as a result in advances in treatment, it remains the most common cancer and the second leading cause of cancer death among women owing to the high and increasing incidence (*136*). According to the American Cancer Society, the lifetime risk of a woman developing breast cancer is 13% or almost one in eight. Patients with noninvasive cancers have a good treatment prognosis and have a high survival rate. However, invasive cancer moves into the lymphatic system and metastasizes to regional lymph nodes, which help it spread to different tissues. A breast-conserving surgical approach benefits some patients, while others receive neoadjuvant (presurgery) chemotherapy or hormone therapy to downstage large tumors, thus potentially allowing conservative surgery (*136, 137*). Moreover, adjuvant therapy (chemotherapy given after surgery) is used to eradicate micrometasteses, to improve survival rate and to delay tumor recurrence (*138*). Anthracycline (doxorubicin or epirubicin) and taxane-based agents have been the standard chemotherapy for breast cancer (*139, 140*). However, many patients with progressive disease are resistant to these treatment options. Response to chemotherapy is used as a prognostic factor (*141-143*).

Advanced breast cancer has a cure rate of less than 5%, so the usual treatment goal is prolongation of survival or improvement of quality of life (*144, 145*). Furthermore, only women with estrogen receptor-positive and node-negative tumors (not spread to the lymph nodes) receive hormone therapy (such as the antagonist tamoxifen, Nolvadex®) with no extra benefit from chemotherapy (*146, 147*). Chemotherapeutic agents have well-known serious side effects, and anti-hormone therapy can lead to uterine cancer (in the case of tamoxifen) (*138*). Additionally some patients can be administered trastuzumab (Herceptin®) only if their tumors overexpress the HER-2 receptor (Human epidermal growth factor receptor 2), limiting its general use. Currently, some breast cancers do not have combination therapies that address their particular molecular status. These facts make p53 an attractive target due to its tumor suppressor ability and its ability to act independently as an apoptotic factor in any cell type. In fact, patients with mutations in p53 are advised to have annual screening for breast cancer because most current anti-cancer agents are more efficacious when p53 is not mutated (*148*). It is considered the worst prognosis if p53 is inactivated (*149*). Knowledge of key molecular changes such as p53 cytoplasmic mislocalization (or mutation) in cancer cells will facilitate further advances in treatment of breast cancer by developing strategies that involve p53.

An excellent target for p53 therapy is inflammatory breast carcinoma, an aggressive and deadly form of breast cancer (*150*) which has mislocalized or mutated p53 (*151*) and therefore should readily respond to this type of therapy. Cell lines with mislocalized or mutated p53 will serve as the starting point for these studies. While this

application focuses on breast cancer, this approach is applicable to *all* types of cancers involving p53 mislocalization, nuclear exclusion, mutation, or inactivation.

Mitochondrial targeting of p53 is further discussed in this section. The targeting is investigated by fusing the p53 protein with different mitochondrial signals (MTSs) to achieve an improved in activity. In addition, mutations in the p53 nuclear localization signal (NLS) were introduced to reduce nuclear targeting and hence increase the protein's availability in the cytoplasm to target the mitochondria. A p53 "protein switch" was also used to exploit our emerging technology where ligand induction leads to nuclear accumulation of the target protein (*152*). Taking advantage of both the protein switch and the p53/MDM2 degradation pathway, a proteasomal protein switch was developed. Proteasomal switch can be used to target aberrant proteins in the cytoplasm and send them for degradation upon ligand addition.

The p53 Protein

The p53 protein plays a pivotal role in suppression of most cancers (*153-156*). Half of all tumors have mutant p53 (*157*), while inactivity of p53 defines the majority of the remaining cancer cases (*158*). Additionally, the apoptotic pathways of p53 have now been clearly delineated. Nuclear accumulation of p53 is essential for its transcriptional activities leading to induction of proteins involved in both the intrinsic and extrinsic apoptotic pathways (*158-160*). Also, p53 triggers a nontranscriptionally mediated intrinsic apoptotic response if delivered to the mitochondria (*161, 162*). Indeed, p53 has emerged as a "master switch" for cancer prevention (*154*) and is being actively pursued as the ultimate cancer therapeutic target.

Tumor suppressor p53, a 393 amino acid residue is encoded by the TP53 gene, located at 17p13 (reviewed in (*163*)). The protein can be divided into three regions (Figure 1.5): an acidic N-terminal region (codons 1-101), a DNA binding domain (DBD, codons 102-292), and a basic C-terminal region (codons 293-393). The acidic N-terminal region contains a transactivation acidic domain (codons 1-42), a MDM2 binding domain (MBD, codons 17-28), and a proline-rich domain (PRD, codons 63-97). The basic C-terminal region contains three NLSs (codons 305-322 most active NLS), a tetramerization domain (TD, codons 323-3356), and a negative regulatory region (codons 363-393). p53 functions as a transcription factor in many cases, regulating expression of genes involved in apoptosis, cell-cycle arrest, senescence, DNA repair, cell survival, and genomic stability, among others (*163-165*). Therefore it is difficult to predict its response to chemotherapy in cancer patients (*166*). Under normal growth conditions, p53 is rapidly degraded (*167*). In reaction to the p53 response, many autoregulatory feedback loops are triggered. Of the ten positive or negative feedback loops, six act through the MDM2 (mouse double minute 2) protein to regulate p53 activity (reviewed in (*168*)). MDM2 is the main cellular antagonist of p53, and is responsible for nuclear exclusion and proteasomal degradation of p53 (*169*). Disruption of the p53-MDM2 complex has become an important cancer therapeutic target (*170*). Upon stress, p53 is activated as a transcription factor by posttranslational modifications through stress signals such as DNA damage, UV, carcinogens, nitric oxide, heat or cold shock, or hypoxia (*171*). These modifications increase the half-life of the protein from 6-20 min to hours resulting in a 3- to 10- fold concentration increase. In addition, they enhance the ability of p53 to bind to specific DNA sequences and to promote their transcription (*168*). As a result of cell

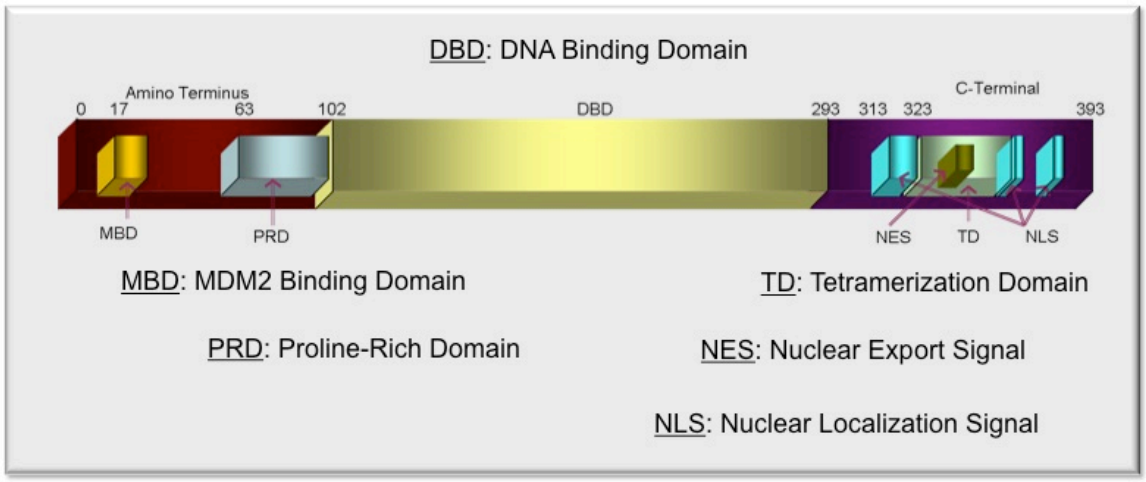


Figure 1.5. The full-length p53. The 393 amino acids of p53 are divided into amino terminus, DNA binding domain (DBD), and C-terminal region. The amino terminus contains the MDM2 binding domain (MBD) and proline-rich domain (PRD). The C-terminal region contains three nuclear localization signals (NLSs), a nuclear export signal (NES), and a tetramerization domain (TD).

signaling, p53 rapidly accumulates in the nucleus, which is essential for regulating cell cycle arrest, DNA repair, and apoptosis (*159, 160*). In general, posttranslational modifications (ubiquitination, phosphorylation, methylation, sumoylation, neddylation, glycosylation and acetylation) are known to regulate p53 activity in terms of stability and function (reviewed in (*172-174*)). Although p53 is tightly regulated by these modifications, the fact remains that gene therapy with p53 has been successful (*175-177*), thus circumventing the need to deliberately include these highly complex (and often unresolved) issues in gene therapy of p53. Tetramerization of p53 through its TD is also crucial for its transcriptional activity, protein-protein interactions, and degradation (*178*).

Apoptosis through p53 occurs via two distinct signaling pathways (*154*): the extrinsic pathway through death receptors and the intrinsic pathway through the mitochondria (Figure 1.6). The extrinsic pathway of p53-mediated apoptosis occurs via induction of Fas, DR5, and PERP genes. These encode 3 transmembrane "death receptors" that belong to the tumor necrosis factor receptor family. After Fas binding to Fas-L (ligand) (*179*), DR5 binding to TRAIL (ligand) (*180*), or activation of PERP (ligand unknown) (*181, 182*), these proteins induce caspase-8 mediated apoptosis. The intrinsic pathway of p53-mediated apoptosis centers around the Bcl-2 family of proteins, which contain both pro- and anti-apoptotic members. Pro-apoptotic members include Bax, Noxa, and PUMA (*154*). The genes encoding these proteins are targets for p53 transcriptional activation/induction. Bax, Noxa, and PUMA all act at the mitochondria to activate apoptosis (reviewed in (*183-185*)). A link between the extrinsic and intrinsic pathways is provided by Bid protein (*186, 187*). Bid is also another pro-apoptotic

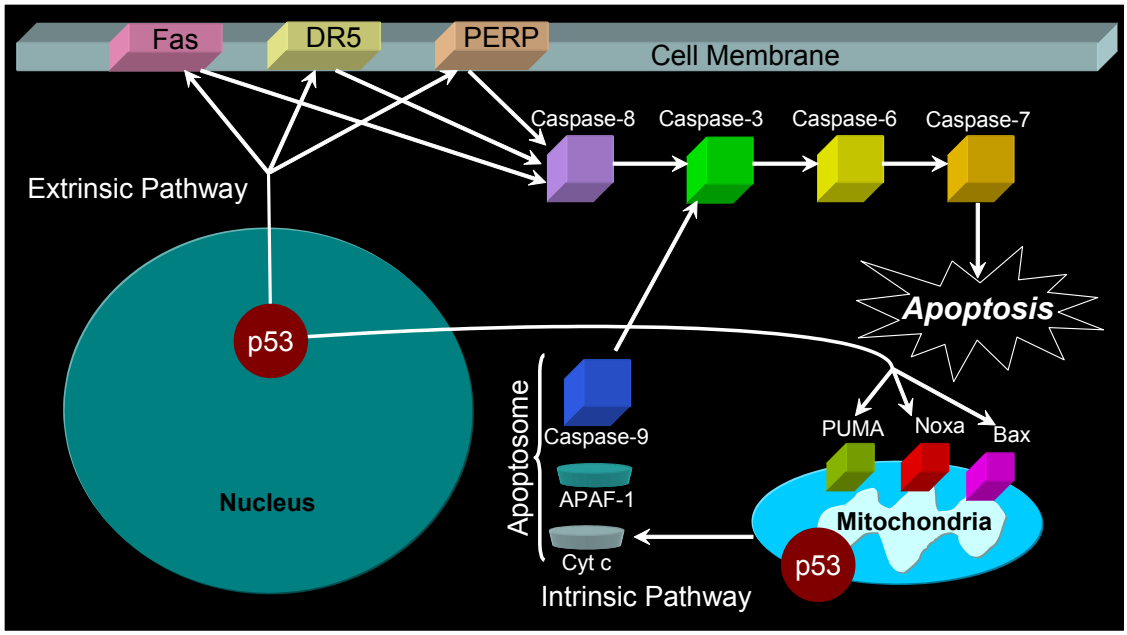


Figure 1.6. The extrinsic and intrinsic apoptosis pathways. Caspases 8 and 9 are initiator caspases, while caspases 3, 6, and 7 are effector caspases. Extrinsic pathway- p53 induces genes encoding three transmembrane proteins: Fas, DR5, and PERP; activation of any leads to caspase-8 activation and triggering of apoptosis. Intrinsic pathway- p53 targets a key subset of the Bcl-2 family of genes including Bax, Noxa, and PUMA. These proteins participate in the release of cytochrome c from the mitochondria, which in turn with APAF-1 (a p53 regulated gene) activate caspase-9, triggering apoptosis. Cytochrome c, APAF-1, and caspase-9 form the apoptosome.

member of the Bcl-2 family that is cleaved and activated by caspase-8. Cleaved Bid undergoes myristoylation, and then acts at the mitochondria to induce apoptosis. p53 itself also acts directly at the mitochondria to induce a rapid apoptotic response that occurs prior to p53 target gene activation. Via its DNA binding domain, p53 interacts with Bcl-XL (an anti-apoptotic) and Bcl-2 to promote permeabilization of the outer mitochondrial membrane (*161, 162*). Clearly p53 plays an important role in preventing cancers from arising in a cell population by activating apoptotic pathways.

Somatic TP53 missense mutations occur in at least 50% of all cancers, while inactivity of p53 is in the majority of the remaining cancer cases (*158, 188, 189*). In cancer cells, overexpressed mutant p53 (with mutations mainly in DBD) abolishes its transcription factor activity (*189-191*). Mutant p53 proteins are generally more stable because of the inability of MDM2 to be induced and therefore mutant p53 accumulates in the nucleus of tumor cells (*190*). The protein also becomes inactivated through different mechanisms, some of which are through elevation of p53 inhibitor MDM2 and silencing of key p53 co-activators such as ARF (*192*). Moreover, p53 is prevented from functioning as a suppressor through abnormal cytoplasmic sequestration and inhibition of its access to the nucleus, suggesting that the sequestration of p53 in the cytoplasm may represent a nonmutational mechanism of p53 inactivation (*151, 193, 194*). Mislocalization of p53 occurs in 37% of breast cancer (*151*) while p53 mutations occur in 30% of breast cancer (*195-197*). Parc (p53-associated Parkin-like cytoplasmic protein) plays an important role in binding to the C-terminus of p53 in the cytoplasm and prevents transport into the nucleus (*198*). Studies have shown that mutant p53 is associated with resistance to chemotherapy (*199, 200*). Indeed, an exogenous C-terminal p53 peptide was

able to induce nuclear accumulation of endogenous p53 by competition for binding to Parc (*201*). These studies provide compelling evidence that regulating the shuttling of p53 between the cytoplasm and the nucleus or the mitochondria offers a controlled therapeutic approach to re-direct the mislocalized proteins to their correct cellular compartment.

Localization of p53 to the mitochondria can also induce apoptosis via an extranuclear death function of p53 (*162, 202*), reflecting p53-induced apoptosis in the absence of transactivation of genes or protein synthesis (*203, 204*). A small but highly reproducible fraction of p53 translocates to the mitochondria at the onset of p53-dependent apoptosis. The majority of mitochondrial p53 is found in the membranous compartment while a fraction of p53 is in a complex with the mitochondrial import motor hsp70 (*162*). Apoptosis ensues after critical changes in the mitochondria occur including collapse of the inner transmembrane potential, disruption of electron transport and ATP production, oxidative stress, permeability transition pore opening, and mitochondrial swelling with outer membrane rupture. When the outer mitochondrial membrane undergoes permeabilization, cytochrome c is released from the intramembranous space into the cytosol triggering apoptosis via the apoptosome (Figure 1.6) (*105, 205, 206*). In cancer cells, inducing the mitochondrial outer membrane permeabilization constitutes a therapeutic goal (*207*). Translocation of p53 to the mitochondria triggers the release of cytochrome c and procaspase-3 activation by forming inhibitory complexes with anti-apoptotic Bcl-XL and Bcl-2 proteins through its DBD (residues 239-248) and inducing oligomerization of the BH123 effector protein Bak (*161, 202, 208*). Such events precede the nuclear translocation of p53 and its target gene activation (*161, 209*). When p53 is

targeted to the mitochondria at high levels, it induces apoptosis as efficiently as nuclear p53 by itself without requiring additional DNA damage. In cancer cells with endogenous missense mutants of p53, these mutants are not able to bind to Bcl-2 proteins due to mutation in the DBD, demonstrating the importance of DBD in mitochondrial apoptosis (*208*). Additionally during hydrogen peroxide-induced apoptosis, the protective effect of the small hsp αB-crystallin is due to its ability to bind to p53, sequester it in the cytoplasm, and prevent translocation to the mitochondria (*210-212*). These last two examples underscore the importance of directing functional p53 to the mitochondria for certain apoptotic events. The TD of p53, on the other hand, is not essential for mitochondrial function, since targeting a truncated version of p53 (residues 1-305) without the TD to the mitochondria can still induce apoptosis (*162*).

Mitochondrial targeting of p53 can be achieved by adding a MTS specific for the outer membrane. To further enhance mitochondrial localization, the strong NLS in p53 (*213*) can also be inactivated. The mitochondrial targeting of p53 is further explored in Chapter 5.

Mitochondria in Cancer Cells

Mitochondria are crucial in regulating the intrinsic apoptotic pathway in response to DNA damage, growth factor withdrawal, hypoxia, or oncogene deregulation (*214, 215*). The intrinsic pathway is initiated by outer mitochondrial membrane permeabilization, which facilitates the release of potent death factors from the intramembranous space into the cytosol (*206*). These factors including cytochrome c can activate the caspases responsible for apoptosis. Mitochondrial dysfunction plays a central

role in the development and progression of tumors (*109-111*). It could initiate apoptosis and necrosis resulting in organ failure.

Large molecules cross the outer and inner membranes through the TOM complex (translocase of the outer membrane) and the TIM complex (translocase of the inner membrane), respectively. The TOM complex contains two groups of receptor subunits; a group that recognizes proteins destined for import (Tom70 and Tom20), and a group that assists the transfer of the protein into the intramembranous space (Tom40, Tom22, Tom7, Tom6, and Tom5) (*216-221*). Proteins translocate the inner membrane via TIM22, which targets the inner membrane, and TIM23, which targets the matrix (*218, 221*). A protein needs to have a mitochondrial targeting signal (MTS) in order for it to be recognized by either the TOM or TIM complexes. MTSs are usually positively charged N-terminal cleavable peptides (15-40 residues in length). The signal forms an amphipathic α-helix, which is proteolyticaly cleaved by a mitochondrial peptidase in the matrix (*217, 222*).

p53 primarily targets the Bcl2 (B-cell leukemia/lymphoma-2) family proteins in the mitochondria and either enhance their pro-death function or inhibit their survival function. These proteins can be grouped into pro- or anti- apoptotic subfamilies, depending on their modular content of BH1 (Bcl2 homology), BH2, BH3, and BH4 domains (*223*). They can be classified into three classes: BH1234 (such as Bcl-XL and Bcl2), BH123 (such as Bax and Bak), and BH3 only (such as Bad, tBid, and Puma) (Figure 1.7). The first class (BH1234) contains anti-apoptotic proteins, which stabilize the mitochondrial outer membrane via binding to and inhibiting the pro-apoptotic members of the second class (BH123) via their hydrophobic BH123 binding pocket (*224*). The third class (BH3 only) contains key transducers of death signals and can be divided

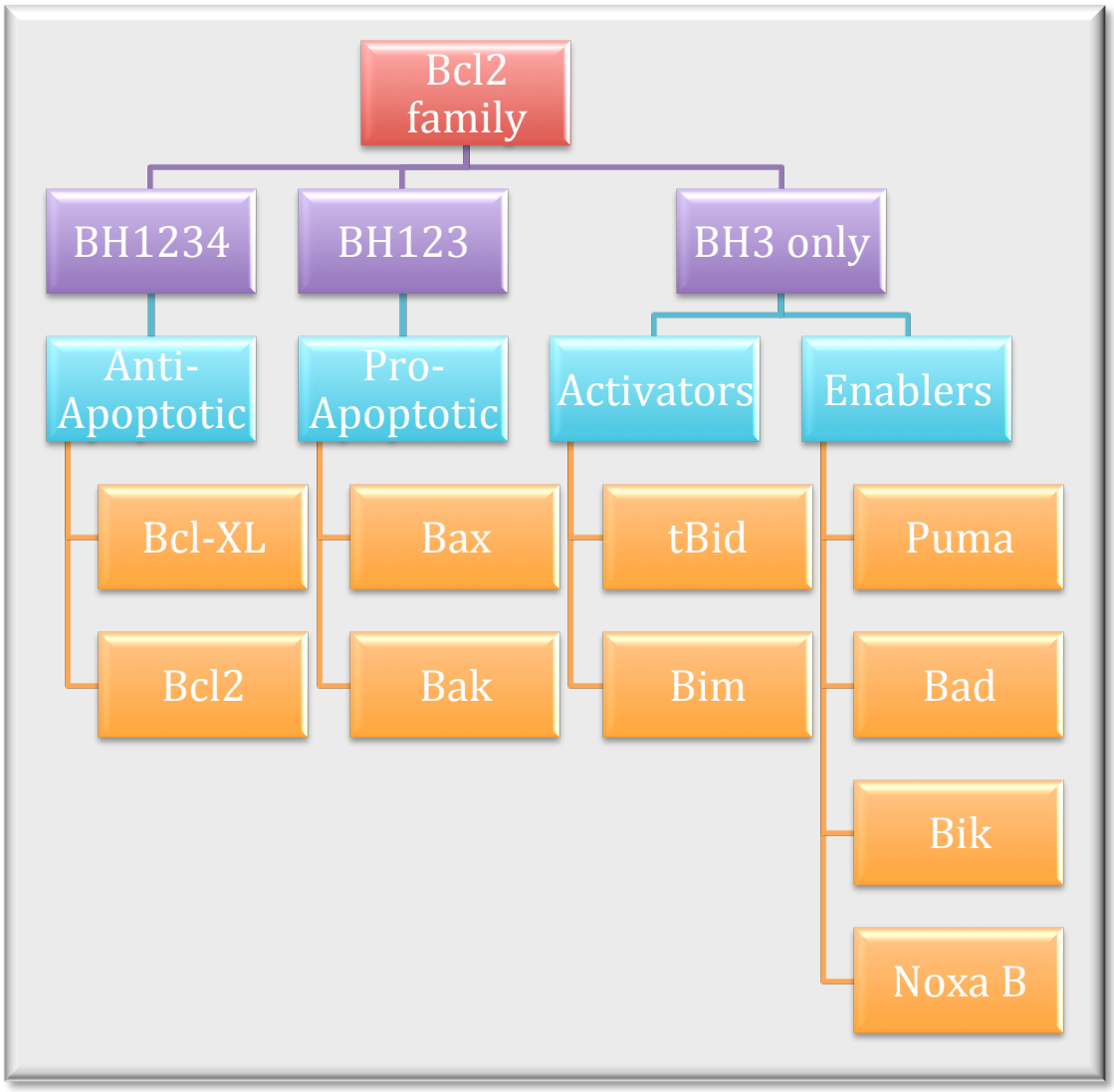


Figure 1.7. The Bcl2 family proteins are grouped according to their modular content of Bcl2 homology (BH). There are BH1234 (anti-apoptotic proteins), BH123 (pro-apoptotic proteins), and BH3 only (activators and enablers).

into two subgroups: activators (such as tBid) and enablers (such as Puma) (*225*). tBid activates Bax and Bak by binding to them and inducing their oligomerization (*225*). Puma promotes mitochondrial outer membrane permeabilization via binding to and deactivating Bcl-XL and Bcl2 and hence freeing Bak and Bax (*224*). p53 acts similar to Puma in inducing apoptosis when targeted to the mitochondria (*202*). It has also been reported that p53 increases the binding between Puma and Bcl-XL by changing the BH3 binding region of Bcl-XL (*223*). p53 also acts similar to tBid by activating and binding directly to Bak and Bax to induce their oligomerization (*226-228*).

Targeting the Nucleus

In 1962, it was demonstrated that nucleocytoplasmic exchange occurs via pores embedded in the nuclear envelope (*229*). These 125 MDa nuclear pore complexes (NPCs) manage protein trafficking, both active and passive transport in and out of the nucleus, accommodating ~1000 translocation events per second (*230-232*). They have a tripartite structure: eight cytoplasmic filaments, a central transport, and a nuclear basket, constructed from approximately 100 nucleoporins (specific proteins that make up the NPC) (*233-236*). The long cytoplasmic filaments (cytoplasmic fibrils) projecting into the cytoplasm interact with karyopherins (family of transport receptors) and deflect non-shuttling proteins (*237-240*). The central transport is a cylindrical intramembrane transporter (central aqueous channel) anchored to the nuclear membrane via eight spokes. Smaller molecules (less than ~40 kDa) transport via passive diffusion due to its 9 nm diameter (*241-243*), whereas larger molecules (>40 kDa and up to 60 MDa) are transported via amino acid signals, which are recognized by specific import and export

receptors (*244-248*). The nuclear basket consists of eight long fibrils projecting into the nucleoplasm, which are connected distally by a ring.

Proteins containing nuclear localization signals (NLSs) are recognized by import receptors. Classical NLSs are monopartite with a single stretch of amino acids similar to the NLS found in SV-40 large tumor antigen (PKKKRKV; critical residues underlined) (*249*). Bipartite (two short amino acid sequences with a spacer in between) NLSs have also been recognized in proteins like nucleoplasmin (KRPAATKKAGQAKKKKLDK) (*250*). NLSs in proteins are recognized and imported into the nucleus by the importin α/β heterodimer. Importin α has the NLS binding site while importin β mediates the translocation of the importin α-NLS cargo complex into the nucleus through the NPC (*244, 251*). The cargo is freed in the nucleoplasm upon RanGTP binding to importin β, which along to importin α are recycled back to the cytoplasm [reviewed in (*236*)]. Conversely, the leucine-rich nuclear export signals (NESs) in the presence of RanGTP are recognized by the classical export receptor CRM1, which mediates the translocation to the cytoplasm (*252*). In the cytoplasm, RanGAP catalyzes the hydrolysis of a phosphate bond in the RanGTP dissociating the complex and freeing the cargo. Classical NESs are usually 8-11 amino acids in length with hydrophobic residues, leucines in particular. Commonly, NES is $LX_{(1-3)}LX_{(2-3)}LXJ$ (where L is leucine, X is a spacer and J is leucine, valine or isoleucine) (*253-255*).

Our lab has also designed a ligand inducible protein switch to control targeting of therapeutic proteins to the nucleus. Tagging EGFP with a strong NES, a ligand-inducible NLS (*256*), and a dexamethasone-specific ligand binding domain (LBD) (*257*) facilitate its bi-directional trafficking capability. The LBD is taken from a nuclear receptor, the

glucocorticoid receptor (GR). LBDs cause nuclear translocation of full-length receptors according to the dose of ligand (*253, 258*). Upon ligand binding, these nuclear receptors undergo a conformational change allowing the NLS to be exposed causing nuclear localization (*259*). The GR LBD used in our studies contains a point mutation (C656G) that makes it 10 times more sensitive to the agonist dexamethasone (Dex) (*258*). Our lab has previously shown that enhanced green fluorescent protein (EGFP)-tagged protein switch translocates from the cytoplasm to the nucleus in a dose-dependent manner (*4, 152*). The protein switch can be engineered with a dimerization domain of a protein of interest which will allow it to "capture" cytoplasmically mislocalized endogenous protein (*236*). This protein switch-mislocalized protein complex can then be directed (by addition of ligand) to its correct compartment, the nucleus.

The elegance of the protein switch is that it imitates proteins found in nature. Many proteins in signal transduction pathways are capable of binding to protein partners in the cell, moving them to a new location and/or altering their function. For example, the p14/19 ARF protein is capable of binding to MDM2, resulting in decreased degradation of p53 by MDM2 (*260*). p14/19 ARF, with its nucleolar targeting signal, is also capable of re-directing the p14/19 ARF-MDM2 complex to the nucleolus (*168, 261*). Like nature, the protein switch was designed to regulate cellular proteins by changing their location and hence their function. The protein switch has the advantage of being regulated by externally added ligand (*4, 152, 236*).

An alternative proteasomal protein switch that utilizes MDM2 mediated degradation of p53 was discovered in the course of these studies. MDM2 binds to p53 and results in proteasomal degradation of p53. This mechanism is described in Chapter 6.

Proteasomal Targeting

Proteins marked with a death signal, a chain of polyubiquitin, are targeted to the proteasome for degradation (*262*). The proteins are further degraded into amino acids, which are then used in synthesizing new proteins. Proteasomes are found in both the nucleus and cytoplasm. The proteasome consists of the 20S proteasomal core and two 19S regulatory caps. The caps contain multiple ATPase active sites and ubiquitin binding sites. Proteasomal targeting has been studied to either inhibit the proteasome for inflammatory disease and cancer, or activate it for neurodegenerative diseases (*263, 264*).

The ubiquitin-proteasome system is crucial for proteasomal degradation of proteins (*265*). The proteasome recognizes ubiquitinated proteins for degradation. An ubiquitin-activating enzyme (E1) first hydrolyzes ATP and is linked to an ubiquitin molecule via a thioester bond. Next, the ubiquitin molecule is transferred to an ubiquitin-ligating enzyme (E2). In the last step, an ubiquitin ligase (E3) binds to a specific protein (substrate) and helps the transfer of ubiquitin from E2 to a lysine residue in the target protein. E3 binding to the substrate is considered the rate-limiting step in protein degradation (*263*). The polyubiquitin chains marks the protein for proteasomal degradation.

The ubiquitin transfer from E2 to the targeted protein can occur in two ways: via the RING (really interesting new gene) domain of E3s or via the HECT (homologous to the E6-AP carboxy terminus) domain of E3s (*266*). MDM2 containing the RING domain is the ubiquitin ligase (E3) responsible for binding to and ubiquitinating p53 accompanied by Ubc5, E2 ubiquitin conjugating enzyme (*267*). The main lysine residues in p53 that

are ubiquitinated by MDM2 are found on the carboxy terminus, K370, K372, K373, K381, K381, and K386 (*268*).

Gene Therapy

For the induction of apoptosis in breast cancer, constructs encoding p53 have been used in gene therapy. Generally, gene therapy is the insertion of a gene (a well-defined DNA sequence) into cells' nuclei to produce specific therapeutic proteins to correct a cellular dysfunction or to provide a new cellular function (*269*). If a specific gene contains a defect, the corresponding expressed protein usually loses its normal function. Once the key protein(s) and mechanisms involved are identified in a disease, gene therapy could be used as a treatment therapy. Identifying genes and proteins involved in diseases has been greatly improved due to the Human Genome Project and the Hap Map Project (*270*). Clinical trials using gene therapy have been focusing on certain diseases such as cancer, cardiovascular disease, inherited monogenic disease, and infectious disease (mainly HIV). The largest target for gene therapy clinical trials is cancer, which is a more complex disease involving mutations in a number of genes (*271, 272*). Since gene therapy is not designed to deliver a vast amount of different genes, certain critical proteins involved in signaling cascades are targeted. The first clinically approved gene therapy product, approved only in China, was the delivery of the tumor suppressor p53 via an adenovirus under the brand name Gendicine™. This drug was approved by China's State Food and Drug Administration (SFDA) in 2004 for head and neck squamous cell cancer (*273-275*). It also showed promising results in liver cancer, lung cancer, and other advanced liver tumors (*274*). It is noteworthy that Gendicine™

was approved by the SFDA on the basis of tumor shrinkage and not on the basis of lifetime extension of treated patients as required are by the U.S. FDA and the European Medicine Agency (EMA) (*270*).

Gene therapy using wt p53 has had some successes for many types of cancers, including lung, ovarian, glioma, etc. (*175-177, 276-283*). More recently, attempts to use p53 targeted to the mitochondria have also shown promise (*207, 284*). Nuclear and mitochondrial p53 have been shown in one study to be equipotent (*162*), but have different mechanisms of action for apoptosis. Our mitochondrial p53 showed enhanced mitochondrial targeting (and is designed to cause apoptosis) due to rationally designed alterations/additions to the gene to be delivered (Chapter 5). While the actual delivery of these gene-therapeutics is not the focus of this work, ultimately these constructs could be delivered virally or nonvirally as genetically encoded products.

Statement of Objectives

The long-term objectives of this project are to harness different methods of targeting multiple organelles in a cell to lead to better drug delivery and improvement in disease therapy. This study describes the optimization of protein (adiponectin) delivery to activate certain mechanisms in the mitochondria, the designing of a small molecule (MitoE) to specifically target the mitochondria, and utilization of gene therapy to control the targeting of proteins (such as p53) to the mitochondria, nucleus and proteasome.

Two disease models were investigated to explore the different subcellular targeting of therapeutics. For cardiovascular disease, controlled delivery of adiponectin and mitochondrial targeting of vitamin E were tested to reduce the oxidative stress in

endothelial cells. Adiponectin reduced the oxidative stress via cAMP pathway while maintaining its activity after it was released from the biodegradable triblock copolymer (Chapter 3). In addition, vitamin E conjugated to TPP showed recovery of endothelial dysfunction by reducing ROS after its accumulation in the mitochondria (Chapter 4). For breast cancer, p53 was targeted to the mitochondria (Chapter 5), nucleus and proteasome (Chapter 6). Mitochondrial and nuclear targeting were achieved by fusing MTS and NLS respectively. However, proteasomal targeting was accomplished via fusing protein switch to p53 followed by ligand addition. Nuclear and mitochondrial p53 are responsible for inducing apoptosis in breast cancer cells whereas proteasomal targeting is still under investigation for possible targeting of oncogenic proteins. These approaches lead to the hypothesis that *targeting specific organelles in a cell will lead to better drug delivery and improvements in disease therapy*. In this study four major hypotheses were proposed, with corresponding aims, as follows:

Hypothesis 1: Cardiac mitochondrial oxidative stress can be reversed by local controlled delivery of adiponectin using a triblock copolymer.

Aim: To examine the effect of the released adiponectin on mitochondrial ROS and perform mechanistic studies.

Hypothesis 2: Specific mitochondrial targeting of vitamin E can reduce ROS production in the cardiac mitochondria.

Aim: To conjugate vitamin E to TPP to increase mitochondrial targeting and determine the ability of modified antioxidants to reduce mitochondrial ROS.

Hypothesis 3: Fusing MTS to p53 will increase its mitochondrial targeting and apoptotic potential.

Aim: To prove that a nuclear-localization deficient version of p53 engineered with an improved mitochondrial targeting signal will trigger apoptosis via the intrinsic apoptotic pathway.

Hypothesis 4: A p53-protein switch will localize to the proteasome upon ligand addition.

Aim: To create localization controllable protein construct to the proteasome that could be used as a protein switch for degrading oncogenic proteins.

These hypotheses and specific aims have been discussed sequentially in Chapters 3-6. Chapter 2 discusses the different approaches for subcellular delivery. The results in the studies described in Chapters 2 and 3 have been published in peer-reviewed journals (*123, 23*). Chapter 5 has been submitted to *Molecular Pharmaceutics* while Chapter 4 is complete and will be submitted to the *Journal of Organic Chemistry*.

References

1. Dixon, A. S., Kakar, M., Schneider, K. M., Constance, J. E., Paullin, B. C., and Lim, C. S. (2009) Controlling subcellular localization to alter function: Sending oncogenic Bcr-Abl to the nucleus causes apoptosis, *J. Control. Release 140*, 245-249.

2. Davis, J. R., Kakar, M., and Lim, C. S. (2007) Controlling protein compartmentalization to overcome disease, *Pharm. Res. 24*, 17-27.

3. Kakar, M., Davis, J. R., Kern, S. E., and Lim, C. S. (2007) Optimizing the protein switch: altering nuclear import and export signals, and ligand binding domain, *J. Control. Release 120*, 220-232.

4. Kanwal, C., Mu, S., Kern, S. E., and Lim, C. S. (2004) Bidirectional on/off switch for controlled targeting of proteins to subcellular compartments, *J. Control. Release 98*, 379-393.

5. Chiuve, S. E., McCullough, M. L., Sacks, F. M., and Rimm, E. B. (2006) Healthy lifestyle factors in the primary prevention of coronary heart disease among men: benefits among users and nonusers of lipid-lowering and antihypertensive medications, *Circulation 114*, 160-167.

6. Haffner, S., and Taegtmeyer, H. (2003) Epidemic obesity and the metabolic syndrome, *Circulation 108*, 1541-1545.

7. Rolo, A. P., Teodoro, J. S., and Palmeira, C. M. (2011) Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis, *Free Radic. Biol. Medicine [Epub ahead of print] 22064361*.

8. Tsutsui, H., Kinugawa, S., and Matsushima, S. (2011) Oxidative stress and heart failure, *Am. J. Physiol. 301*, H2181-2190.

9. Szasz, T., Thakali, K., Fink, G. D., and Watts, S. W. (2007) A comparison of arteries and veins in oxidative stress: producers, destroyers, function, and disease, *Exp. Biol. Med. 232*, 27-37.

10. Mueller, C. F., Laude, K., McNally, J. S., and Harrison, D. G. (2005) ATVB in focus: redox mechanisms in blood vessels, *Arterioscler. Thromb. Vasc. Biol. 25*, 274-278.

11. Li, J. M., and Shah, A. M. (2004) Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology, *Am. J. Physiol. Regul. Integr. Comp. Physiol. 287*, R1014-1030.

12. Miller, F. J., Jr., Gutterman, D. D., Rios, C. D., Heistad, D. D., and Davidson, B. L. (1998) Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis, *Circ. Res. 82*, 1298-1305.

13. Cai, H. (2005) Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences, *Cardiovasc. Res. 68*, 26-36.

14. Paz, K., Hemi, R., LeRoith, D., Karasik, A., Elhanany, E., Kanety, H., and Zick, Y. (1997) A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation, *J. Biol. Chem. 272*, 29911-29918.

15. Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2002) Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes, *Endocr. Rev. 23*, 599-622.

16. Evans, J. L., Maddux, B. A., and Goldfine, I. D. (2005) The molecular basis for oxidative stress-induced insulin resistance, *Antioxidants Redox Signaling 7*, 1040-1052.

17. Mendez, J. I., Nicholson, W. J., and Taylor, W. R. (2005) SOD isoforms and signaling in blood vessels: evidence for the importance of ROS compartmentalization, *Arterioscler. Thromb. Vasc. Biol. 25*, 887-888.

18. Atsumi, H., Kitada, M., Kanasaki, K., and Koya, D. (2011) Reversal of redox-dependent inhibition of diacylglycerol kinase by antioxidants in mesangial cells exposed to high glucose, *Mol. Med. Reports 4*, 923-927.

19. Volchegorskii, I. A., Rassokhina, L. M., Miroshnichenko, I. Y., Mester, K. M., Novoselov, P. N., and Astakhova, T. V. (2011) Effect of pro- and antioxidants on insulin sensitivity and glucose tolerance, *Bulletin Experim. Biol. Med. 150*, 327-332.

20. Muratovska, A., Lightowlers, R. N., Taylor, R. W., Wilce, J. A., and Murphy, M. P. (2001) Targeting large molecules to mitochondria, *Advanc. Drug Delivery Rev. 49*, 189-198.

21. Morin, D., Hauet, T., Spedding, M., and Tillement, J. (2001) Mitochondria as target for antiischemic drugs, *Advanc. Drug Deliv. Rev. 49*, 151-174.

22. Modica-Napolitano, J. S., and Aprille, J. R. (2001) Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells, *Advanc. Drug Deliv. Rev. 49*, 63-70.

23. Mossalam, M., Jeong, J. H., Abel, E. D., Kim, S. W., and Kim, Y. H. (2008) Reversal of oxidative stress in endothelial cells by controlled release of adiponectin, *J. Control. Release 130*, 234-237.

24. Choi, S., Baudys, M., and Kim, S. W. (2004) Control of blood glucose by novel GLP-1 delivery using biodegradable triblock copolymer of PLGA-PEG-PLGA in type 2 diabetic rats, *Pharm. Res. 21*, 827-831.

25. Choi, S., and Kim, S. W. (2003) Controlled release of insulin from injectable biodegradable triblock copolymer depot in ZDF rats, *Pharm. Res. 20*, 2008-2010.

26. Chen, S., Pieper, R., Webster, D. C., and Singh, J. (2005) Triblock copolymers: synthesis, characterization, and delivery of a model protein, *Intl. J. Pharmac. 288*, 207-218.

27. Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S., and Beal, M. F. (2004) Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species, *J. Neuroscience 24*, 7779-7788.

28. Quinn, M. T., and Gauss, K. A. (2004) Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases, *J. Leukocyte Biol. 76*, 760-781.

29. Sheu, S. S., Nauduri, D., and Anders, M. W. (2006) Targeting antioxidants to mitochondria: a new therapeutic direction, *Biochim. et Biophys. Acta. 1762*, 256-265.

30. Fridovich, I. (1995) Superoxide radical and superoxide dismutases, *Annual Rev. Biochem. 64*, 97-112.

31. Reubsaet, F. A., Veerkamp, J. H., Bukkens, S. G., Trijbels, J. M., and Monnens, L. A. (1988) Acyl-CoA oxidase activity and peroxisomal fatty acid oxidation in rat tissues, *Biochimica. et. Biophysica. Acta. 958*, 434-442.

32. Winterbourn, C. C., Vissers, M. C., and Kettle, A. J. (2000) Myeloperoxidase, *Current Opin. Hematol. 7*, 53-58.

33. Seshiah, P. N., Weber, D. S., Rocic, P., Valppu, L., Taniyama, Y., and Griendling, K. K. (2002) Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators, *Circ. Res. 91*, 406-413.

34. McNally, J. S., Davis, M. E., Giddens, D. P., Saha, A., Hwang, J., Dikalov, S., Jo, H., and Harrison, D. G. (2003) Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress, *Am. J. Physiol. Heart Circ. Physiol. 285*, H2290-2297.

35. Linder, N., Rapola, J., and Raivio, K. O. (1999) Cellular expression of xanthine oxidoreductase protein in normal human tissues, *Lab Invest. 79*, 967-974.

36. Jones, S. P., and Bolli, R. (2006) The ubiquitous role of nitric oxide in cardioprotection, *J. Mol. Cellul. Cardiol. 40*, 16-23.

37. Shiva, S., Sack, M. N., Greer, J. J., Duranski, M., Ringwood, L. A., Burwell, L., Wang, X., MacArthur, P. H., Shoja, A., Raghavachari, N., Calvert, J. W., Brookes, P. S., Lefer, D. J., and Gladwin, M. T. (2007) Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer, *J. Experim. Med. 204*, 2089-2102.

38. Motoshima, H., Wu, X., Mahadev, K., and Goldstein, B. J. (2004) Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL, *Biochem. Biophys. Res. Commun. 315*, 264-271.

39. Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P., and Pritchard, K. A., Jr. (1998) Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors, *Proc. Natl. Acad. Sci. USA 95*, 9220-9225.

40. Reznick, R. M., and Shulman, G. I. (2006) The role of AMP-activated protein kinase in mitochondrial biogenesis, *J. Physiol. 574*, 33-39.

41. Reznick, R. M., Zong, H., Li, J., Morino, K., Moore, I. K., Yu, H. J., Liu, Z. X., Dong, J., Mustard, K. J., Hawley, S. A., Befroy, D., Pypaert, M., Hardie, D. G., Young, L. H., and Shulman, G. I. (2007) Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis, *Cell Metabol. 5*, 151-156.

42. Yamagishi, S. I., Edelstein, D., Du, X. L., Kaneda, Y., Guzman, M., and Brownlee, M. (2001) Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A, *J. Biol. Chem. 276*, 25096-25100.

43. Smith, R. A., Porteous, C. M., Coulter, C. V., and Murphy, M. P. (1999) Selective targeting of an antioxidant to mitochondria, *Eur. J. Biochem. 263*, 709-716.

44. Lago, F., Dieguez, C., Gomez-Reino, J., and Gualillo, O. (2007) Adipokines as emerging mediators of immune response and inflammation, *Natre. Clinic. Prac. Rheumatol. 3*, 716-724.

45. Ouchi, N., Kihara, S., Funahashi, T., Matsuzawa, Y., and Walsh, K. (2003) Obesity, adiponectin and vascular inflammatory disease, *Current Opin. Lipidol. 14*, 561-566.

46. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuzawa, Y. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity, *Biochem Biophys Res Commun 257*, 79-83.

47. Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., and Tataranni, P. A. (2001) Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia, *J. Clin. Endocrinol. Metab. 86*, 1930-1935.

48. Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T., and Matsuzawa, Y. (2000) Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients, *Arterioscler. Thromb. Vasc. Biol. 20*, 1595-1599.

49. Lindsay, R. S., Funahashi, T., Hanson, R. L., Matsuzawa, Y., Tanaka, S., Tataranni, P. A., Knowler, W. C., and Krakoff, J. (2002) Adiponectin and development of type 2 diabetes in the Pima Indian population, *Lancet 360*, 57-58.

50. Spranger, J., Kroke, A., Mohlig, M., Bergmann, M. M., Ristow, M., Boeing, H., and Pfeiffer, A. F. (2003) Adiponectin and protection against type 2 diabetes mellitus, *Lancet 361*, 226-228.

51. Yamamoto, Y., Hirose, H., Saito, I., Nishikai, K., and Saruta, T. (2004) Adiponectin, an adipocyte-derived protein, predicts future insulin resistance: two-year follow-up study in Japanese population, *J. Clinic. Endocrinol. Metabol. 89*, 87-90.

52. Adamczak, M., Wiecek, A., Funahashi, T., Chudek, J., Kokot, F., and Matsuzawa, Y. (2003) Decreased plasma adiponectin concentration in patients with essential hypertension, *Am. J. Hypertension 16*, 72-75.

53. Matsubara, M., Maruoka, S., and Katayose, S. (2002) Decreased plasma adiponectin concentrations in women with dyslipidemia, *J. Clinic. Endocrinol. Metabol. 87*, 2764-2769.

54. Ouchi, N., Kihara, S., Arita, Y., Maeda, K., Kuriyama, H., Okamoto, Y., Hotta, K., Nishida, M., Takahashi, M., Nakamura, T., Yamashita, S., Funahashi, T., and

Matsuzawa, Y. (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin, *Circulation 100*, 2473-2476.

55. Kumada, M., Kihara, S., Sumitsuji, S., Kawamoto, T., Matsumoto, S., Ouchi, N., Arita, Y., Okamoto, Y., Shimomura, I., Hiraoka, H., Nakamura, T., Funahashi, T., and Matsuzawa, Y. (2003) Association of hypoadiponectinemia with coronary artery disease in men, *Arterioscler. Thromb. Vasc. Biol. 23*, 85-89.

56. Otsuka, F., Sugiyama, S., Kojima, S., Maruyoshi, H., Funahashi, T., Sakamoto, T., Yoshimura, M., Kimura, K., Umemura, S., and Ogawa, H. (2007) Hypoadiponectinemia is associated with impaired glucose tolerance and coronary artery disease in non-diabetic men, *Circulation 71*, 1703-1709.

57. Komura, N., Kihara, S., Sonoda, M., Kumada, M., Fujita, K., Hiuge, A., Okada, T., Nakagawa, Y., Tamba, S., Kuroda, Y., Hayashi, N., Sumitsuji, S., Kawamoto, T., Matsumoto, S., Ouchi, N., Arita, Y., Okamoto, Y., Shimomura, I., Funahashi, T., and Matsuzawa, Y. (2008) Clinical significance of high-molecular weight form of adiponectin in male patients with coronary artery disease, *Circulation 72*, 23-28.

58. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsubara, K. (1996) cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1), *Biochem. Biophys. Res. Commun. 221*, 286-289.

59. Iwaki, M., Matsuda, M., Maeda, N., Funahashi, T., Matsuzawa, Y., Makishima, M., and Shimomura, I. (2003) Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors, *Diabetes 52*, 1655-1663.

60. Davies, G. F., McFie, P. J., Khandelwal, R. L., and Roesler, W. J. (2002) Unique ability of troglitazone to up-regulate peroxisome proliferator-activated receptor-gamma expression in hepatocytes, *J. Pharmacol. Experim. Therap. 300*, 72-77.

61. Campbell, S. E., Stone, W. L., Whaley, S. G., Qui, M., and Krishnan, K. (2003) Gamma (gamma) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (gamma) expression in SW 480 human colon cancer cell lines, *BMC Cancer 3*, 25.

62. Hsieh, C. C., Huang, C. J., and Lin, B. F. (2006) Low and high levels of alpha-tocopherol exert opposite effects on IL-2 possibly through the modulation of PPAR-gamma, IkappaBalpha, and apoptotic pathway in activated splenocytes, *Nutrition 22*, 433-440.

63. De Pascale, M. C., Bassi, A. M., Patrone, V., Villacorta, L., Azzi, A., and Zingg, J. M. (2006) Increased expression of transglutaminase-1 and PPARgamma after

vitamin E treatment in human keratinocytes, *Arch. Biochem. Biophys. 447*, 97-106.

64. Sun, X., and Zemel, M. B. (2007) Calcium and 1,25-dihydroxyvitamin D3 regulation of adipokine expression, *Obesity (Silver Spring) 15*, 340-348.

65. Shen, X. H., Tang, Q. Y., Huang, J., and Cai, W. (2010) Vitamin E regulates adipocytokine expression in a rat model of dietary-induced obesity, *Experim. Biol. Med. 235*, 47-51.

66. Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N. H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., and Kadowaki, T. (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects, *Nature 423*, 762-769.

67. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kadowaki, T. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase, *Nature Med. 8*, 1288-1295.

68. Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P., and Kadowaki, T. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity, *Nature Med. 7*, 941-946.

69. Iwabu, M., Yamauchi, T., Okada-Iwabu, M., Sato, K., Nakagawa, T., Funata, M., Yamaguchi, M., Namiki, S., Nakayama, R., Tabata, M., Ogata, H., Kubota, N., Takamoto, I., Hayashi, Y. K., Yamauchi, N., Waki, H., Fukayama, M., Nishino, I., Tokuyama, K., Ueki, K., Oike, Y., Ishii, S., Hirose, K., Shimizu, T., Touhara, K., and Kadowaki, T. (2010) Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca(2+) and AMPK/SIRT1, *Nature 464*, 1313-1319.

70. Kishida, K., Nagaretani, H., Kondo, H., Kobayashi, H., Tanaka, S., Maeda, N., Nagasawa, A., Hibuse, T., Ohashi, K., Kumada, M., Nishizawa, H., Okamoto, Y., Ouchi, N., Maeda, K., Kihara, S., Funahashi, T., and Matsuzawa, Y. (2003) Disturbed secretion of mutant adiponectin associated with the metabolic syndrome, *Biochem. Biophys. Res. Commun. 306*, 286-292.

71. Pajvani, U. B., Du, X., Combs, T. P., Berg, A. H., Rajala, M. W., Schulthess, T., Engel, J., Brownlee, M., and Scherer, P. E. (2003) Structure-function studies of

the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity, *J. Biol. Chem. 278*, 9073-9085.

72. Shibata, R., Ouchi, N., Kihara, S., Sato, K., Funahashi, T., and Walsh, K. (2004) Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of amp-activated protein kinase signaling, *J. Biol. Chem. 279*, 28670-28674.

73. Ouchi, N., Kobayashi, H., Kihara, S., Kumada, M., Sato, K., Inoue, T., Funahashi, T., and Walsh, K. (2004) Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells, *J. Biol. Chem. 279*, 1304-1309.

74. Kobayashi, H., Ouchi, N., Kihara, S., Walsh, K., Kumada, M., Abe, Y., Funahashi, T., and Matsuzawa, Y. (2004) Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin, *Circ. Res. 94*, e27-31.

75. Croteau-Chonka, D. C., Wu, Y., Li, Y., Fogarty, M. P., Lange, L. A., Kuzawa, C. W., McDade, T. W., Borja, J. B., Luo, J., Abdelbaky, O., Combs, T. P., Adair, L. S., Lange, E. M., and Mohlke, K. L. (2011) Population-specific coding variant underlies genome-wide association with adiponectin level, *Hum. Mol. Genet. [Epub ahead of print] 22010046*.

76. Jalovaara, K., Santaniemi, M., Timonen, M., Jokelainen, J., Kesaniemi, Y. A., Ukkola, O., Keinanen-Kiukaanniemi, S., and Rajala, U. (2008) Low serum adiponectin level as a predictor of impaired glucose regulation and type 2 diabetes mellitus in a middle-aged Finnish population, *Metabolism 57*, 1130-1134.

77. Wu, Y., Li, Y., Lange, E. M., Croteau-Chonka, D. C., Kuzawa, C. W., McDade, T. W., Qin, L., Curocichin, G., Borja, J. B., Lange, L. A., Adair, L. S., and Mohlke, K. L. (2010) Genome-wide association study for adiponectin levels in Filipino women identifies CDH13 and a novel uncommon haplotype at KNG1-ADIPOQ, *Hum. Mol. Genet. 19*, 4955-4964.

78. Pankov Iu, A. (2006) Adiponectin, a new hormone: its role in the pathogenesis of diabetes mellitus, *Vestnik Rossiiskoi Akademii Meditsinskikh Nauk*, 99-104.

79. Thamer, C., Haap, M., Heller, E., Joel, L., Braun, S., Tschritter, O., Haring, H., and Fritsche, A. (2006) Beta cell function, insulin resistance and plasma adiponectin concentrations are predictors for the change of postprandial glucose in non-diabetic subjects at risk for type 2 diabetes, *Horm. Metabol. Res. 38*, 178-182.

80. Goto, T., Teraminami, A., Lee, J. Y., Ohyama, K., Funakoshi, K., Kim, Y. I., Hirai, S., Uemura, T., Yu, R., Takahashi, N., and Kawada, T. (2011) Tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via

activation of adiponectin signaling followed by enhancement of fatty acid oxidation in liver and skeletal muscle in obese-diabetic mice, *J. Nutrition. Biochem. [Epub ahead of print] 21889885.*

81. Chen, H., Montagnani, M., Funahashi, T., Shimomura, I., and Quon, M. J. (2003) Adiponectin stimulates production of nitric oxide in vascular endothelial cells, *J. Biol. Chem. 278*, 45021-45026.

82. Cheng, K. K., Lam, K. S., Wang, Y., Huang, Y., Carling, D., Wu, D., Wong, C., and Xu, A. (2007) Adiponectin-induced endothelial nitric oxide synthase activation and nitric oxide production are mediated by APPL1 in endothelial cells, *Diabetes 56*, 1387-1394.

83. Thomson, M. J., Frenneaux, M. P., and Kaski, J. C. (2009) Antioxidant treatment for heart failure: friend or foe?, *Qjm 102*, 305-310.

84. Mak, S., and Newton, G. E. (2001) The oxidative stress hypothesis of congestive heart failure: radical thoughts, *Chest 120*, 2035-2046.

85. Halliwell, B. (1997) Antioxidants: the basics--what they are and how to evaluate them, *Adv. Pharmacol. 38*, 3-20.

86. Konorev, E. A., Kennedy, M. C., and Kalyanaraman, B. (1999) Cell-permeable superoxide dismutase and glutathione peroxidase mimetics afford superior protection against doxorubicin-induced cardiotoxicity: the role of reactive oxygen and nitrogen intermediates, *Arch. Biochem. Biophys. 368*, 421-428.

87. Malassagne, B., Ferret, P. J., Hammoud, R., Tulliez, M., Bedda, S., Trebeden, H., Jaffray, P., Calmus, Y., Weill, B., and Batteux, F. (2001) The superoxide dismutase mimetic MnTBAP prevents Fas-induced acute liver failure in the mouse, *Gastroenterology 121*, 1451-1459.

88. Laurent, A., Nicco, C., Van Nhieu, J. T., Borderie, D., Chereau, C., Conti, F., Jaffray, P., Soubrane, O., Calmus, Y., Weill, B., and Batteux, F. (2004) Pivotal role of superoxide anion and beneficial effect of antioxidant molecules in murine steatohepatitis, *Hepatol. 39*, 1277-1285.

89. Carr, A., and Frei, B. (1999) Does vitamin C act as a pro-oxidant under physiological conditions?, *FASEB J. 13*, 1007-1024.

90. Ellis, G. R., Anderson, R. A., Lang, D., Blackman, D. J., Morris, R. H., Morris-Thurgood, J., McDowell, I. F., Jackson, S. K., Lewis, M. J., and Frenneaux, M. P. (2000) Neutrophil superoxide anion--generating capacity, endothelial function and oxidative stress in chronic heart failure: effects of short- and long-term vitamin C therapy, *J. Am. College Cardiol. 36*, 1474-1482.

91. Heller, R., Unbehaun, A., Schellenberg, B., Mayer, B., Werner-Felmayer, G., and Werner, E. R. (2001) L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin, *J. Biol. Chem. 276*, 40-47.

92. Libby, P., and Aikawa, M. (2002) Vitamin C, collagen, and cracks in the plaque, *Circulation 105*, 1396-1398.

93. Okamoto, K. (2002) Vitamin C intake and apolipoproteins in a healthy elderly Japanese population, *Prev. Med. 34*, 364-369.

94. Golbidi, S., Badran, M., and Laher, I. (2011) Diabetes and alpha lipoic Acid, *Frontiers Pharmacol. 2*, 69.

95. Azzi, A., Gysin, R., Kempna, P., Munteanu, A., Villacorta, L., Visarius, T., and Zingg, J. M. (2004) Regulation of gene expression by alpha-tocopherol, *J. Biol. Chem. 385*, 585-591.

96. Paolisso, G., D'Amore, A., Giugliano, D., Ceriello, A., Varricchio, M., and D'Onofrio, F. (1993) Pharmacologic doses of vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetic patients, *Am. J. Clinic. Nutrition 57*, 650-656.

97. Paolisso, G., Di Maro, G., Galzerano, D., Cacciapuoti, F., Varricchio, G., Varricchio, M., and D'Onofrio, F. (1994) Pharmacological doses of vitamin E and insulin action in elderly subjects, *Am. J. Clinic. Nutrition 59*, 1291-1296.

98. Mayer-Davis, E. J., Costacou, T., King, I., Zaccaro, D. J., and Bell, R. A. (2002) Plasma and dietary vitamin E in relation to incidence of type 2 diabetes: The Insulin Resistance and Atherosclerosis Study (IRAS), *Diabetes Care 25*, 2172-2177.

99. Caballero, B. (1993) Vitamin E improves the action of insulin, *Nutrition Rev. 51*, 339-340.

100. Vinayaga Moorthi, R., Bobby, Z., Selvaraj, N., and Sridhar, M. G. (2006) Vitamin E protects the insulin sensitivity and redox balance in rat L6 muscle cells exposed to oxidative stress, *Inter. J. Clinic. Chem. 367*, 132-136.

101. Rosen, P., Nawroth, P. P., King, G., Moller, W., Tritschler, H. J., and Packer, L. (2001) The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society, *Diabetes Metab. Res. Rev. 17*, 189-212.

102. West, I. C. (2000) Radicals and oxidative stress in diabetes, *Diabet. Med. 17*, 171-180.

103. Miller, E. R., 3rd, Pastor-Barriuso, R., Dalal, D., Riemersma, R. A., Appel, L. J., and Guallar, E. (2005) Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality, *Annals Intern. Med. 142*, 37-46.

104. Papa, S., and Skulachev, V. P. (1997) Reactive oxygen species, mitochondria, apoptosis and aging, *Mol. Cellular Biochem. 174*, 305-319.

105. Green, D. R., and Reed, J. C. (1998) Mitochondria and apoptosis, *Science 281*, 1309-1312.

106. Pinkoski, M. J., Waterhouse, N. J., and Green, D. R. (2006) Mitochondria, apoptosis and autoimmunity, *Current Direct. Autoimmun. 9*, 55-73.

107. Horobin, R. W., Trapp, S., and Weissig, V. (2007) Mitochondriotropics: a review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria, *J. Control. Release 121*, 125-136.

108. Weissig, V., and Torchilin, V. P. (2001) Drug and DNA delivery to mitochondria, *Adv. Drug Deliv. Rev. 49*, 1-2.

109. Newmeyer, D. D., and Ferguson-Miller, S. (2003) Mitochondria: releasing power for life and unleashing the machineries of death, *Cell 112*, 481-490.

110. Detmer, S. A., and Chan, D. C. (2007) Functions and dysfunctions of mitochondrial dynamics, *Nature Rev. 8*, 870-879.

111. Wallace, D. C. (2005) Mitochondria and cancer: Warburg addressed, *Cold Spring Harbor Symp. Quant. Biol. 70*, 363-374.

112. Johnson, D. T., Harris, R. A., French, S., Blair, P. V., You, J., Bemis, K. G., Wang, M., and Balaban, R. S. (2007) Tissue heterogeneity of the mammalian mitochondrial proteome, *Am. J. Physiol. 292*, C689-697.

113. Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., and Sheu, S. S. (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle, *Am. J. Physiol. 287*, C817-833.

114. Abid, M. R., Schoots, I. G., Spokes, K. C., Wu, S. Q., Mawhinney, C., and Aird, W. C. (2004) Vascular endothelial growth factor-mediated induction of manganese superoxide dismutase occurs through redox-dependent regulation of forkhead and IkappaB/NF-kappaB, *J. Biol. Chem. 279*, 44030-44038.

115. Zhang, H. J., Drake, V. J., Xu, L., Hu, J., Domann, F. E., Oberley, L. W., and Kregel, K. C. (2002) Redox regulation of adenovirus-induced AP-1 activation by

overexpression of manganese-containing superoxide dismutase, *J. Virol. 76*, 355-363.

116. Li, S., Yan, T., Yang, J. Q., Oberley, T. D., and Oberley, L. W. (2000) The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase, *Cancer Res. 60*, 3927-3939.

117. Warner, B. B., Stuart, L., Gebb, S., and Wispe, J. R. (1996) Redox regulation of manganese superoxide dismutase, *Am. J. Physiol. 271*, L150-158.

118. Schiavone, J. R., and Hassan, H. M. (1988) The role of redox in the regulation of manganese-containing superoxide dismutase biosynthesis in Escherichia coli, *J. Biol. Chem. 263*, 4269-4273.

119. Ross, E. A., Koo, L. C., and Moberly, J. B. (1997) Low whole blood and erythrocyte levels of glutathione in hemodialysis and peritoneal dialysis patients, *Am. J. Kidney Diseases 30*, 489-494.

120. Rocha, M., Hernandez-Mijares, A., Garcia-Malpartida, K., Banuls, C., Bellod, L., and Victor, V. M. (2010) Mitochondria-targeted antioxidant peptides, *Current Pharmac. Des. 16*, 3124-3131.

121. Ross, M. F., Kelso, G. F., Blaikie, F. H., James, A. M., Cocheme, H. M., Filipovska, A., Da Ros, T., Hurd, T. R., Smith, R. A., and Murphy, M. P. (2005) Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology, *Biochem. 70*, 222-230.

122. Adlam, V. J., Harrison, J. C., Porteous, C. M., James, A. M., Smith, R. A., Murphy, M. P., and Sammut, I. A. (2005) Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury, *FASEB J. 19*, 1088-1095.

123. Mossalam, M., Dixon, A. S., and Lim, C. S. (2010) Controlling subcellular delivery to optimize therapeutic effect, *Ther. Deliv. 1*, 169-193.

124. James, A. M., Sharpley, M. S., Manas, A. R., Frerman, F. E., Hirst, J., Smith, R. A., and Murphy, M. P. (2007) Interaction of the mitochondria-targeted antioxidant MitoQ with phospholipid bilayers and ubiquinone oxidoreductases, *J. Biol. Chem. 282*, 14708-14718.

125. Filipovska, A., Kelso, G. F., Brown, S. E., Beer, S. M., Smith, R. A., and Murphy, M. P. (2005) Synthesis and characterization of a triphenylphosphonium-conjugated peroxidase mimetic. Insights into the interaction of ebselen with mitochondria, *J. Biol. Chem. 280*, 24113-24126.

126. Smith, R. A., Porteous, C. M., Gane, A. M., and Murphy, M. P. (2003) Delivery of bioactive molecules to mitochondria in vivo, *PNAS USA 100*, 5407-5412.

127. Yu, L., Chang, G. T., Zhang, H., and Ding, J. D. (2008) Injectable block copolymer hydrogels for sustained release of a PEGylated drug, *Intl. J. Pharmac. 348*, 95-106.

128. Chen, S., and Singh, J. (2008) Controlled release of growth hormone from thermosensitive triblock copolymer systems: In vitro and in vivo evaluation, *Intl. J. Pharmac. 352*, 58-65.

129. Jeong, B., Bae, Y. H., and Kim, S. W. (2000) In situ gelation of PEG-PLGA-PEG triblock copolymer aqueous solutions and degradation thereof, *J. Biomed. Mat. Res. 50*, 171-177.

130. Bezemer, J. M., Radersma, R., Grijpma, D. W., Dijkstra, P. J., van Blitterswijk, C. A., and Feijen, J. (2000) Microspheres for protein delivery prepared from amphiphilic multiblock copolymers. 1. Influence of preparation techniques on particle characteristics and protein delivery, *J. Control. Release 67*, 233-248.

131. Van de Weert, M., Hennink, W. E., and Jiskoot, W. (2000) Protein instability in poly(lactic-co-glycolic acid) microparticles, *Pharm. Res. 17*, 1159-1167.

132. Qiao, M., Chen, D., Hao, T., Zhao, X., Hu, H., and Ma, X. (2008) Injectable thermosensitive PLGA-PEG-PLGA triblock copolymers-based hydrogels as carriers for interleukin-2, *Pharmazie 63*, 27-30.

133. Jeong, B., Bae, Y. H., and Kim, S. W. (2000) Drug release from biodegradable injectable thermosensitive hydrogel of PEG-PLGA-PEG triblock copolymers, *J. Control. Release 63*, 155-163.

134. Kim, Y. J., Choi, S., Koh, J. J., Lee, M., Ko, K. S., and Kim, S. W. (2001) Controlled release of insulin from injectable biodegradable triblock copolymer, *Pharm. Res. 18*, 548-550.

135. Choi, S., Baudys, M., and Kim, S. W. (2004) Control of blood glucose by novel GLP-1 delivery using biodegradable triblock copolymer of PLGA-PEG-PLGA in type 2 diabetic rats, *Pharm. Res. 21*, 827-831.

136. Turner, N. C., and Jones, A. L. (2008) Management of breast cancer--part I, *BMJ 337*, a421.

137. Kaufmann, M., von Minckwitz, G., and Rody, A. (2005) Preoperative (neoadjuvant) systemic treatment of breast cancer, *Breast 14*, 576-581.

138. Turner, N. C., and Jones, A. L. (2008) Management of breast cancer--Part II, *BMJ 337*, a540.

139. De Laurentiis, M., Cancello, G., D'Agostino, D., Giuliano, M., Giordano, A., Montagna, E., Lauria, R., Forestieri, V., Esposito, A., Silvestro, L., Pennacchio, R., Criscitiello, C., Montanino, A., Limite, G., Bianco, A. R., and De Placido, S. (2008) Taxane-based combinations as adjuvant chemotherapy of early breast cancer: a meta-analysis of randomized trials, *J. Clin. Oncol. 26*, 44-53.

140. (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials, *Lancet 365*, 1687-1717.

141. Guarneri, V., Broglio, K., Kau, S. W., Cristofanilli, M., Buzdar, A. U., Valero, V., Buchholz, T., Meric, F., Middleton, L., Hortobagyi, G. N., and Gonzalez-Angulo, A. M. (2006) Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors, *J. Clin. Oncol. 24*, 1037-1044.

142. Michaelis, L. C., and Ratain, M. J. (2006) Measuring response in a post-RECIST world: from black and white to shades of grey, *Nature Rev. Cancer 6*, 409-414.

143. Bertheau, P., Lerebours, F., Mounier, N., de Roquancourt, A., Espie, M., Clot, P., Servant, J. M., Misset, J. L., Marty, M., and Janin, A. (2005) Prognostic significance of a combined clinicopathologic score for response to primary systemic therapy in locally advanced breast cancer, *Oncol. Rep. 14*, 513-520.

144. Takahashi, S., Ito, Y., Hatake, K., and Sugimoto, Y. (2006) Gene therapy for breast cancer. --Review of clinical gene therapy trials for breast cancer and MDR1 gene therapy trial in Cancer Institute Hospital, *Breast Cancer 13*, 8-15.

145. Hortobagyi, G. N. (1998) Treatment of breast cancer, *N. Engl. J. Med. 339*, 974-984.

146. Paik, S., Tang, G., Shak, S., Kim, C., Baker, J., Kim, W., Cronin, M., Baehner, F. L., Watson, D., Bryant, J., Costantino, J. P., Geyer, C. E., Jr., Wickerham, D. L., and Wolmark, N. (2006) Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer, *J. Clin. Oncol. 24*, 3726-3734.

147. Paik, S., Shak, S., Tang, G., Kim, C., Baker, J., Cronin, M., Baehner, F. L., Walker, M. G., Watson, D., Park, T., Hiller, W., Fisher, E. R., Wickerham, D. L., Bryant, J., and Wolmark, N. (2004) A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer, *N. Engl. J. Med. 351*, 2817-2826.

148. Robson, M., and Offit, K. (2007) Clinical practice. Management of an inherited predisposition to breast cancer, *N. Engl. J. Med. 357*, 154-162.

149. O'Connor, P. M., Jackman, J., Bae, I., Myers, T. G., Fan, S., Mutoh, M., Scudiero, D. A., Monks, A., Sausville, E. A., Weinstein, J. N., Friend, S., Fornace, A. J., Jr., and Kohn, K. W. (1997) Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents, *Cancer Res. 57*, 4285-4300.

150. Chittoor, S. R., and Swain, S. M. (1998) Locally advanced breast cancer: Role of medical oncology, In *The Breast: Comprehensive Management of Benign and Malignant Diseases*. (I., B. K., and M., C. E., Eds.) 2nd ed., W.B. Saunders Company, Philadelphia.

151. Moll, U. M., Riou, G., and Levine, A. J. (1992) Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion, *PNAS USA 89*, 7262-7266.

152. Kakar, M., Davis, J. R., Kern, S. E., and Lim, C. S. (2007) Optimizing the protein switch: altering nuclear import and export signals, and ligand binding domain, *J. Control. Release 120*, 220-232.

153. Bullock, A. N., and Fersht, A. R. (2001) Rescuing the function of mutant p53, *Nat. Rev. Cancer 1*, 68-76.

154. Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. (2003) Apoptosis - the p53 network, *J. Cell Sci. 116*, 4077-4085.

155. Hupp, T. R., Lane, D. P., and Ball, K. L. (2000) Strategies for manipulating the p53 pathway in the treatment of human cancer, *Biochem. J. 352 Pt 1*, 1-17.

156. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) Regulation of the specific DNA binding function of p53, *Cell 71*, 875-886.

157. Bykov, V. J., Issaeva, N., Shilov, A., Hultcrantz, M., Pugacheva, E., Chumakov, P., Bergman, J., Wiman, K. G., and Selivanova, G. (2002) Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound, *Nat. Med. 8*, 282-288.

158. Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001) Regulation and function of the p53 tumor suppressor protein, *Curr. Opin. Cell Biol. 13*, 332-337.

159. Shaulsky, G., Goldfinger, N., Tosky, M. S., Levine, A. J., and Rotter, V. (1991) Nuclear localization is essential for the activity of p53 protein, *Oncogene 6*, 2055-2065.

160. Baptiste, N., and Prives, C. (2004) p53 in the cytoplasm: a question of overkill?, *Cell 116*, 487-489.

161. Erster, S., Mihara, M., Kim, R. H., Petrenko, O., and Moll, U. M. (2004) In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation, *Mol. Cell. Biol. 24*, 6728-6741.

162. Marchenko, N. D., Zaika, A., and Moll, U. M. (2000) Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling, *J. Biol. Chem. 275*, 16202-16212.

163. Vousden, K. H., and Lane, D. P. (2007) p53 in health and disease, *Nat. Rev. Mol. Cell Biol. 8*, 275-283.

164. Miyashita, T., and Reed, J. C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, *Cell 80*, 293-299.

165. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., and et al. (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis, *Cancer Res. 54*, 1169-1174.

166. Bertheau, P., Espie, M., Turpin, E., Lehmann, J., Plassa, L. F., Varna, M., Janin, A., and de The, H. (2008) TP53 status and response to chemotherapy in breast cancer, *Pathobiology 75*, 132-139.

167. Gudkov, A. V. (2002) Converting p53 from a killer into a healer, *Nat. Med. 8*, 1196-1198.

168. Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops, *Oncogene 24*, 2899-2908.

169. Ringshausen, I., O'Shea, C. C., Finch, A. J., Swigart, L. B., and Evan, G. I. (2006) Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo, *Cancer Cell 10*, 501-514.

170. Moll, U. M., and Petrenko, O. (2003) The MDM2-p53 interaction, *Mol. Cancer Res. 1*, 1001-1008.

171. Royds, J. A., and Iacopetta, B. (2006) p53 and disease: when the guardian angel fails, *Cell Death Differ. 13*, 1017-1026.

172. Bode, A. M., and Dong, Z. (2004) Post-translational modification of p53 in tumorigenesis, *Nat. Rev. Cancer 4*, 793-805.

173. Brooks, C. L., and Gu, W. (2003) Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation, *Curr. Opin. Cell Biol. 15*, 164-171.

174. Lacroix, M., Toillon, R. A., and Leclercq, G. (2006) p53 and breast cancer, an update, *Endocr. Relat. Cancer 13*, 293-325.

175. Buller, R. E., Runnebaum, I. B., Karlan, B. Y., Horowitz, J. A., Shahin, M., Buekers, T., Petrauskas, S., Kreienberg, R., Slamon, D., and Pegram, M. (2002) A phase I/II trial of rAd/p53 (SCH 58500) gene replacement in recurrent ovarian cancer, *Cancer Gene Ther. 9*, 553-566.

176. Lang, F. F., Bruner, J. M., Fuller, G. N., Aldape, K., Prados, M. D., Chang, S., Berger, M. S., McDermott, M. W., Kunwar, S. M., Junck, L. R., Chandler, W., Zwiebel, J. A., Kaplan, R. S., and Yung, W. K. (2003) Phase I trial of adenovirus-mediated p53 gene therapy for recurrent glioma: biological and clinical results, *J. Clin. Oncol. 21*, 2508-2518.

177. Cristofanilli, M., Krishnamurthy, S., Guerra, L., Broglio, K., Arun, B., Booser, D. J., Menander, K., Van Wart Hood, J., Valero, V., and Hortobagyi, G. N. (2006) A nonreplicating adenoviral vector that contains the wild-type p53 transgene combined with chemotherapy for primary breast cancer: safety, efficacy, and biologic activity of a novel gene-therapy approach, *Cancer 107*, 935-944.

178. Chene, P. (2001) The role of tetramerization in p53 function, *Oncogene 20*, 2611-2617.

179. Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R., and Weissberg, P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis, *Science 282*, 290-293.

180. Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and el-Deiry, W. S. (1997) KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene, *Nat. Genet. 17*, 141-143.

181. Attardi, L. D., Reczek, E. E., Cosmas, C., Demicco, E. G., McCurrach, M. E., Lowe, S. W., and Jacks, T. (2000) PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family, *Genes Dev. 14*, 704-718.

182. Davies, L., Gray, D., Spiller, D., White, M. R., Damato, B., Grierson, I., and Paraoan, L. (2008) P53 apoptosis mediator PERP: localization, function and caspase activation in uveal melanoma, *J. Cell Mol. Med. 13*, 1995-2007.

183. Adams, J. M., and Cory, S. (1998) The Bcl-2 protein family: arbiters of cell survival, *Science 281*, 1322-1326.

184. Ploner, C., Kofler, R., and Villunger, A. (2008) Noxa: at the tip of the balance between life and death, *Oncogene 27 Suppl 1*, S84-92.

185. Yu, J., Wang, Z., Kinzler, K. W., Vogelstein, B., and Zhang, L. (2003) PUMA mediates the apoptotic response to p53 in colorectal cancer cells, *PNAS USA 100*, 1931-1936.

186. Gross, A. (2006) BID as a double agent in cell life and death, *Cell Cycle 5*, 582-584.

187. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis, *Cell 94*, 491-501.

188. Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S. V., Hainaut, P., and Olivier, M. (2007) Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database, *Hum. Mut. 28*, 622-629.

189. Soussi, T., Ishioka, C., Claustres, M., and Beroud, C. (2006) Locus-specific mutation databases: pitfalls and good practice based on the p53 experience, *Nat. Rev. Cancer 6*, 83-90.

190. Soussi, T. (2007) p53 alterations in human cancer: more questions than answers, *Oncogene 26*, 2145-2156.

191. Soussi, T., and Wiman, K. G. (2007) Shaping genetic alterations in human cancer: the p53 mutation paradigm, *Cancer Cell 12*, 303-312.

192. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Surfing the p53 network, *Nature 408*, 307-310.

193. Moll, U. M., LaQuaglia, M., Benard, J., and Riou, G. (1995) Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors, *PNAS USA 92*, 4407-4411.

194. Flamini, G., Curigliano, G., Ratto, C., Astone, A., Ferretti, G., Nucera, P., Sofo, L., Sgambato, A., Boninsegna, A., Crucitti, F., and Cittadini, A. (1996) Prognostic significance of cytoplasmic p53 overexpression in colorectal cancer. An immunohistochemical analysis, *Eur. J. Cancer 32A*, 802-806.

195. Levine, A. J., Momand, J., and Finlay, C. A. (1991) The p53 tumour suppressor gene, *Nature 351*, 453-456.

196. Hinds, P. W., Finlay, C. A., Quartin, R. S., Baker, S. J., Fearon, E. R., Vogelstein, B., and Levine, A. J. (1990) Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes, *Cell Growth Differ. 1*, 571-580.

197. Turpin, E., Bieche, I., Bertheau, P., Plassa, L. F., Lerebours, F., de Roquancourt, A., Olivi, M., Espie, M., Marty, M., Lidereau, R., Vidaud, M., and de The, H. (2002) Increased incidence of ERBB2 overexpression and TP53 mutation in inflammatory breast cancer, *Oncogene 21*, 7593-7597.

198. Kastan, M. B., and Zambetti, G. P. (2003) Parc-ing p53 in the cytoplasm, *Cell 112*, 1-2.

199. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents, *Cell 74*, 957-967.

200. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994) p53 status and the efficacy of cancer therapy in vivo, *Science 266*, 807-810.

201. Ostermeyer, A. G., Runko, E., Winkfield, B., Ahn, B., and Moll, U. M. (1996) Cytoplasmically sequestered wild-type p53 protein in neuroblastoma is relocated to the nucleus by a C-terminal peptide, *PNAS USA 93*, 15190-15194.

202. Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) p53 has a direct apoptogenic role at the mitochondria, *Mol. Cell. 11*, 577-590.

203. Caelles, C., Helmberg, A., and Karin, M. (1994) p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes, *Nature 370*, 220-223.

204. Gao, C., and Tsuchida, N. (1999) Activation of caspases in p53-induced transactivation-independent apoptosis, *Jpn. J. Cancer Res. 90*, 180-187.

205. Reed, J. C. (1997) Cytochrome c: can't live with it--can't live without it, *Cell 91*, 559-562.

206. Green, D. R., and Evan, G. I. (2002) A matter of life and death, *Cancer Cell 1*, 19-30.

207. Galluzzi, L., Morselli, E., Kepp, O., Tajeddine, N., and Kroemer, G. (2008) Targeting p53 to mitochondria for cancer therapy, *Cell Cycle 7*, 1949-1955.

208. Tomita, Y., Marchenko, N., Erster, S., Nemajerova, A., Dehner, A., Klein, C., Pan, H., Kessler, H., Pancoska, P., and Moll, U. M. (2006) WT p53, but not tumor-

derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization, *J. Biol. Chem. 281*, 8600-8606.

209. Zhao, Y., Chaiswing, L., Velez, J. M., Batinic-Haberle, I., Colburn, N. H., Oberley, T. D., and St Clair, D. K. (2005) p53 translocation to mitochondria precedes its nuclear translocation and targets mitochondrial oxidative defense protein-manganese superoxide dismutase, *Cancer Res. 65*, 3745-3750.

210. Liu, S., Li, J., Tao, Y., and Xiao, X. (2007) Small heat shock protein alphaB-crystallin binds to p53 to sequester its translocation to mitochondria during hydrogen peroxide-induced apoptosis, *Biochem. Biophys. Res. Commun. 354*, 109-114.

211. Mao, Y. W., Liu, J. P., Xiang, H., and Li, D. W. (2004) Human alphaA- and alphaB-crystallins bind to Bax and Bcl-X(S) to sequester their translocation during staurosporine-induced apoptosis, *Cell Death Differ. 11*, 512-526.

212. Kamradt, M. C., Chen, F., and Cryns, V. L. (2001) The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation, *J. Biol. Chem. 276*, 16059-16063.

213. Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A., and Rotter, V. (1990) Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis, *Mol. Cell Biol. 10*, 6565-6577.

214. Desai, B. N., Myers, B. R., and Schreiber, S. L. (2002) FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction, *PNAS USA 99*, 4319-4324.

215. Wang, X. (2001) The expanding role of mitochondria in apoptosis, *Genes Dev. 15*, 2922-2933.

216. Endo, T., and Kohda, D. (2002) Functions of outer membrane receptors in mitochondrial protein import, *Biochim Biophys Acta 1592*, 3-14.

217. Dolezal, P., Likic, V., Tachezy, J., and Lithgow, T. (2006) Evolution of the molecular machines for protein import into mitochondria, *Science 313*, 314-318.

218. Rehling, P., Brandner, K., and Pfanner, N. (2004) Mitochondrial import and the twin-pore translocase, *Nat. Rev. Mol. Cell. Biol. 5*, 519-530.

219. Meisinger, C., Ryan, M. T., Hill, K., Model, K., Lim, J. H., Sickmann, A., Muller, H., Meyer, H. E., Wagner, R., and Pfanner, N. (2001) Protein import channel of the outer mitochondrial membrane: a highly stable Tom40-Tom22 core structure

differentially interacts with preproteins, small tom proteins, and import receptors, *Mol. Cell Biol. 21*, 2337-2348.

220. Herrmann, J. M., and Neupert, W. (2000) Protein transport into mitochondria, *Curr. Opin. Microbiol. 3*, 210-214.

221. Koehler, C. M. (2004) New developments in mitochondrial assembly, *Annu. Rev. Cell Dev. Biol. 20*, 309-335.

222. Bohni, P. C., Daum, G., and Schatz, G. (1983) Import of proteins into mitochondria. Partial purification of a matrix-located protease involved in cleavage of mitochondrial precursor polypeptides, *J. Biol. Chem. 258*, 4937-4943.

223. Hagn, F., Klein, C., Demmer, O., Marchenko, N., Vaseva, A., Moll, U. M., and Kessler, H. (2010) BclxL changes conformation upon binding to wild-type but not mutant p53 DNA binding domain, *J. Biol. Chem. 285*, 3439-3450.

224. Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis, *Science 275*, 983-986.

225. Chipuk, J. E., Bouchier-Hayes, L., and Green, D. R. (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario, *Cell Death Differ. 13*, 1396-1402.

226. Pietsch, E. C., Leu, J. I., Frank, A., Dumont, P., George, D. L., and Murphy, M. E. (2007) The tetramerization domain of p53 is required for efficient BAK oligomerization, *Cancer Biol. Ther. 6*, 1576-1583.

227. Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis, *Science 303*, 1010-1014.

228. Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex, *Nat. Cell. Biol. 6*, 443-450.

229. Feldherr, C. M. (1962) The nuclear annuli as pathways for nucleocytoplasmic exchanges, *J. Cell Biol. 14*, 65-72.

230. Harel, A., and Forbes, D. J. (2004) Importin beta: conducting a much larger cellular symphony, *Mol. Cell 16*, 319-330.

231. Wente, S. R. (2000) Gatekeepers of the nucleus, *Science 288*, 1374-1377.

232. Ribbeck, K., and Gorlich, D. (2001) Kinetic analysis of translocation through nuclear pore complexes, *EMBO J. 20*, 1320-1330.

233. Akey, C. W., and Radermacher, M. (1993) Architecture of the Xenopus nuclear pore complex revealed by three-dimensional cryo-electron microscopy, *J. Cell Biol. 122*, 1-19.

234. Allen, T. D., Cronshaw, J. M., Bagley, S., Kiseleva, E., and Goldberg, M. W. (2000) The nuclear pore complex: mediator of translocation between nucleus and cytoplasm, *J. Cell Sci. 113 (Pt 10)*, 1651-1659.

235. Stoffler, D., Fahrenkrog, B., and Aebi, U. (1999) The nuclear pore complex: from molecular architecture to functional dynamics, *Curr. Opin. Cell Biol. 11*, 391-401.

236. Davis, J. R., Kakar, M., and Lim, C. S. (2007) Controlling protein compartmentalization to overcome disease, *Pharm. Res. 24*, 17-27.

237. Seedorf, M., Damelin, M., Kahana, J., Taura, T., and Silver, P. A. (1999) Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase, *Mol. Cell Biol. 19*, 1547-1557.

238. Shah, S., Tugendreich, S., and Forbes, D. (1998) Major binding sites for the nuclear import receptor are the internal nucleoporin Nup153 and the adjacent nuclear filament protein Tpr, *J. Cell Biol. 141*, 31-49.

239. Iovine, M. K., and Wente, S. R. (1997) A nuclear export signal in Kap95p is required for both recycling the import factor and interaction with the nucleoporin GLFG repeat regions of Nup116p and Nup100p, *J. Cell Biol. 137*, 797-811.

240. Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995) The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor, *J. Cell Biol. 131*, 1699-1713.

241. Lyman, S. K., Guan, T., Bednenko, J., Wodrich, H., and Gerace, L. (2002) Influence of cargo size on Ran and energy requirements for nuclear protein import, *J. Cell Biol. 159*, 55-67.

242. Turpin, P., Ossareh-Nazari, B., and Dargemont, C. (1999) Nuclear transport and transcriptional regulation, *FEBS Lett. 452*, 82-86.

243. Paine, P. L., Moore, L. C., and Horowitz, S. B. (1975) Nuclear envelope permeability, *Nature 254*, 109-114.

244. Gorlich, D., and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm, *Annu. Rev. Cell Dev. Biol. 15*, 607-660.

245. Talcott, B., and Moore, M. S. (1999) Getting across the nuclear pore complex, *Trends Cell Biol. 9*, 312-318.

246. Poon, I. K., and Jans, D. A. (2005) Regulation of nuclear transport: central role in development and transformation?, *Traffic 6*, 173-186.

247. Feldherr, C. M., and Akin, D. (1997) The location of the transport gate in the nuclear pore complex, *J. Cell Sci. 110 (Pt 24)*, 3065-3070.

248. Weis, K. (2003) Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle, *Cell 112*, 441-451.

249. Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984) A short amino acid sequence able to specify nuclear location, *Cell 39*, 499-509.

250. Efthymiadis, A., Shao, H., Hubner, S., and Jans, D. A. (1997) Kinetic characterization of the human retinoblastoma protein bipartite nuclear localization sequence (NLS) in vivo and in vitro. A comparison with the SV40 large T-antigen NLS, *J. Biol. Chem. 272*, 22134-22139.

251. Mattaj, I. W., and Englmeier, L. (1998) Nucleocytoplasmic transport: the soluble phase, *Annu. Rev. Biochem. 67*, 265-306.

252. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals, *Cell 90*, 1051-1060.

253. Kanwal, C., Mu, S., Kern, S. E., and Lim, C. S. (2004) Bidirectional on/off switch for controlled targeting of proteins to subcellular compartments, *J. Control. Release 98*, 379-393.

254. Bogerd, H. P., Fridell, R. A., Benson, R. E., Hua, J., and Cullen, B. R. (1996) Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization-selection assay, *Mol. Cell. Biol. 16*, 4207-4214.

255. Kanwal, C., Li, H., and Lim, C. S. (2002) Model system to study classical nuclear export signals, *AAPS PharmSci. 4*, E18.

256. Ylikomi, T., Bocquel, M. T., Berry, M., Gronemeyer, H., and Chambon, P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors, *EMBO J. 11*, 3681-3694.

257. Vegeto, E., Allan, G. F., Schrader, W. T., Tsai, M. J., McDonnell, D. P., and O'Malley, B. W. (1992) The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor, *Cell 69*, 703-713.

258. Htun, H., Barsony, J., Renyi, I., Gould, D. L., and Hager, G. L. (1996) Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera, *PNAS USA 93*, 4845-4850.

259. Wan, Y., Coxe, K. K., Thackray, V. G., Housley, P. R., and Nordeen, S. K. (2001) Separable features of the ligand-binding domain determine the differential subcellular localization and ligand-binding specificity of glucocorticoid receptor and progesterone receptor, *Mol. Endocrinol. 15*, 17-31.

260. Honda, R., and Yasuda, H. (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53, *EMBO J. 18*, 22-27.

261. Lowe, S. W., and Sherr, C. J. (2003) Tumor suppression by Ink4a-Arf: progress and puzzles, *Curr. Opin. Genet. Dev. 13*, 77-83.

262. Nalepa, G., and Wade Harper, J. (2003) Therapeutic anti-cancer targets upstream of the proteasome, *Cancer Treat. Rev. 29 Suppl 1*, 49-57.

263. Nalepa, G., Rolfe, M., and Harper, J. W. (2006) Drug discovery in the ubiquitin-proteasome system, *Nat. Rev. Drug Dis. 5*, 596-613.

264. Dahlmann, B. (2007) Role of proteasomes in disease, *BMC Biochem. 8 Suppl 1*, S3.

265. Vucic, D., Dixit, V. M., and Wertz, I. E. (2011) Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death, *Nat. Rev. Mol. Cell Biol. 12*, 439-452.

266. Pickart, C. M. (2001) Mechanisms underlying ubiquitination, *Annu. Rev. Biochem. 70*, 503-533.

267. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53, *Nature 387*, 296-299.

268. Chan, W. M., Mak, M. C., Fung, T. K., Lau, A., Siu, W. Y., and Poon, R. Y. (2006) Ubiquitination of p53 at multiple sites in the DNA-binding domain, *Mol. Cancer Res. 4*, 15-25.

269. Culver, K. W., and Blaese, R. M. (1994) Gene therapy for cancer, *Trends Genet. 10*, 174-178.

270. Matissek, K. J., Bender, R. R., Davis, J. R., and Lim, C. S. (2011) Choosing Targets for Gene Therapy, *Targets in Gene Therapy In You, Y. Rijeka, Croatia. InTech*, 3-28.

271. Edelstein, M. L., Abedi, M. R., Wixon, J., and Edelstein, R. M. (2004) Gene therapy clinical trials worldwide 1989-2004-an overview, *J. Gene Medicine 6*, 597-602.

272. Edelstein, M. L., Abedi, M. R., and Wixon, J. (2007) Gene therapy clinical trials worldwide to 2007--an update, *J. Gene Med. 9*, 833-842.

273. Shi, J., and Zheng, D. (2009) An update on gene therapy in China, *Curr. Opin. Mol. Ther. 11*, 547-553.

274. Peng, Z. (2005) Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers, *Hum. Gene Ther. 16*, 1016-1027.

275. Xin, H. (2006) Chinese gene therapy. Gendicine's efficacy: hard to translate, *Science 314*, 1233.

276. Wen, S. F., Mahavni, V., Quijano, E., Shinoda, J., Grace, M., Musco-Hobkinson, M. L., Yang, T. Y., Chen, Y., Runnenbaum, I., Horowitz, J., Maneval, D., Hutchins, B., and Buller, R. (2003) Assessment of p53 gene transfer and biological activities in a clinical study of adenovirus-p53 gene therapy for recurrent ovarian cancer, *Cancer Gene Ther. 10*, 224-238.

277. Anderson, S. C., Johnson, D. E., Harris, M. P., Engler, H., Hancock, W., Huang, W. M., Wills, K. N., Gregory, R. J., Sutjipto, S., Wen, S. F., Lofgren, S., Shepard, H. M., and Maneval, D. C. (1998) p53 gene therapy in a rat model of hepatocellular carcinoma: intra-arterial delivery of a recombinant adenovirus, *Clin. Cancer Res. 4*, 1649-1659.

278. Bouvet, M., Ellis, L. M., Nishizaki, M., Fujiwara, T., Liu, W., Bucana, C. D., Fang, B., Lee, J. J., and Roth, J. A. (1998) Adenovirus-mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer, *Cancer Res. 58*, 2288-2292.

279. Zhang, J. F., Hu, C., Geng, Y., Selm, J., Klein, S. B., Orazi, A., and Taylor, M. W. (1996) Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy, *PNAS USA 93*, 4513-4518.

280. Merritt, J. A., Roth, J. A., and Logothetis, C. J. (2001) Clinical evaluation of adenoviral-mediated p53 gene transfer: review of INGN 201 studies, *Semin. Oncol. 28*, 105-114.

281. Buller, R. E., Shahin, M. S., Horowitz, J. A., Runnebaum, I. B., Mahavni, V., Petrauskas, S., Kreienberg, R., Karlan, B., Slamon, D., and Pegram, M. (2002)

Long term follow-up of patients with recurrent ovarian cancer after Ad p53 gene replacement with SCH 58500, *Cancer Gene Ther. 9*, 567-572.

282. (2003) INGN 201: Ad-p53, Ad5CMV-p53, Adenoviral p53, INGN 101, p53 gene therapy--Introgen, RPR/INGN 201, *BioDrugs 17*, 216-222.

283. Roth, J. A., Nguyen, D., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Ferson, D. Z., Hong, W. K., Komaki, R., Lee, J. J., Nesbitt, J. C., Pisters, K. M., Putnam, J. B., Schea, R., Shin, D. M., Walsh, G. L., Dolormente, M. M., Han, C. I., Martin, F. D., Yen, N., Xu, K., Stephens, L. C., McDonnell, T. J., Mukhopadhyay, T., and Cai, D. (1996) Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer, *Nat. Med. 2*, 985-991.

284. Palacios, G., Crawford, H. C., Vaseva, A., and Moll, U. M. (2008) Mitochondrially targeted wild-type p53 induces apoptosis in a solid human tumor xenograft model, *Cell Cycle 7*, 2584-2590.

CHAPTER 2

CONTROLLING SUBCELLULAR DELIVERY TO OPTIMIZE THERAPEUTIC EFFECT

Abstract

This chapter focuses on drug targeting to specific cellular organelles for therapeutic purposes. Drugs can be delivered to all major organelles of the cell (cytosol, endosome/lysosome, nucleus, nucleolus, mitochondria, endoplasmic reticulum, Golgi apparatus, peroxisomes, and proteasomes) where they exert specific effects in those particular subcellular compartments. Delivery can be achieved by chemical (e.g., polymeric) or biological (e.g., signal sequences) means. Unidirectional targeting to individual organelles has proven to be immensely successful for drug therapy. Newer technologies that accommodate multiple signals (protein switch, virus-like delivery systems) mimic nature and allow for a more sophisticated approach to drug delivery. Harnessing different methods of targeting multiple organelles in a cell will lead to better drug delivery and improvements in disease therapy.

Reprinted by permission from *Therapeutic Delivery* 1: 169-93 (2010).
Mossalam M., Dixon A.S., Lim C.S.

M.M. wrote the outline and sections on mitochondria, endoplasmic reticulum, and peroxisome. A.S.D. wrote sections on nucleus, nucleolus and Golgi apparatus. C.S.L. wrote sections on cytosolic delivery, endosome/lysosome, and proteasome.

Introduction

The 1975 discovery of the "Signal Hypothesis" led Gunter Blobel to win a Nobel Prize (*1-3*). Blobel predicted "zip codes" were responsible for targeting certain proteins to subcellular compartments such as the cytoplasm, nucleus, nucleolus, mitochondria, endoplasmic reticulum, Golgi, and peroxisomes (Figure 2.1). The intracellular delivery of a pharmaceutical agent can have a dramatic impact on its therapeutic efficacy. Indeed, precise compartmentalization of certain drugs is necessary for their biological effect. For example, agents intended for gene therapy must be eventually delivered to the nucleus in order for the therapeutic protein to be expressed. Some drugs such as RNA interference, must target the cytosol in order to block the cells' mRNA. In other cases, pro-apoptotic drugs can be selectively targeted to the mitochondria where they exert their actions.

Our previous work has shown that an oncogene can be targeted to a different cellular compartment to completely alter its function. The causative agent of chronic myelogenous leukemia, Bcr-Abl, is normally found in the cytoplasm where it acts as an oncoprotein. However, when targeted to the nucleus (by attaching four nuclear localization signals) it acts as an apoptotic factor (*4*). In this case, targeting a protein to a single organelle can be used to elicit a desired effect such as apoptosis. Further work in our laboratory focuses on controlled localization of proteins to alter function. We have described our "protein switch" technology that allows controlled translocation from the cytoplasm to the nucleus upon addition of ligand (*5-7*). One of the main goals of our research is to imitate the function of proteins found in nature. Many proteins in signal transduction pathways are localized to one compartment initially, bind to protein partners in the cell (or are signaled by other proteins), and change their location in the cell, leading

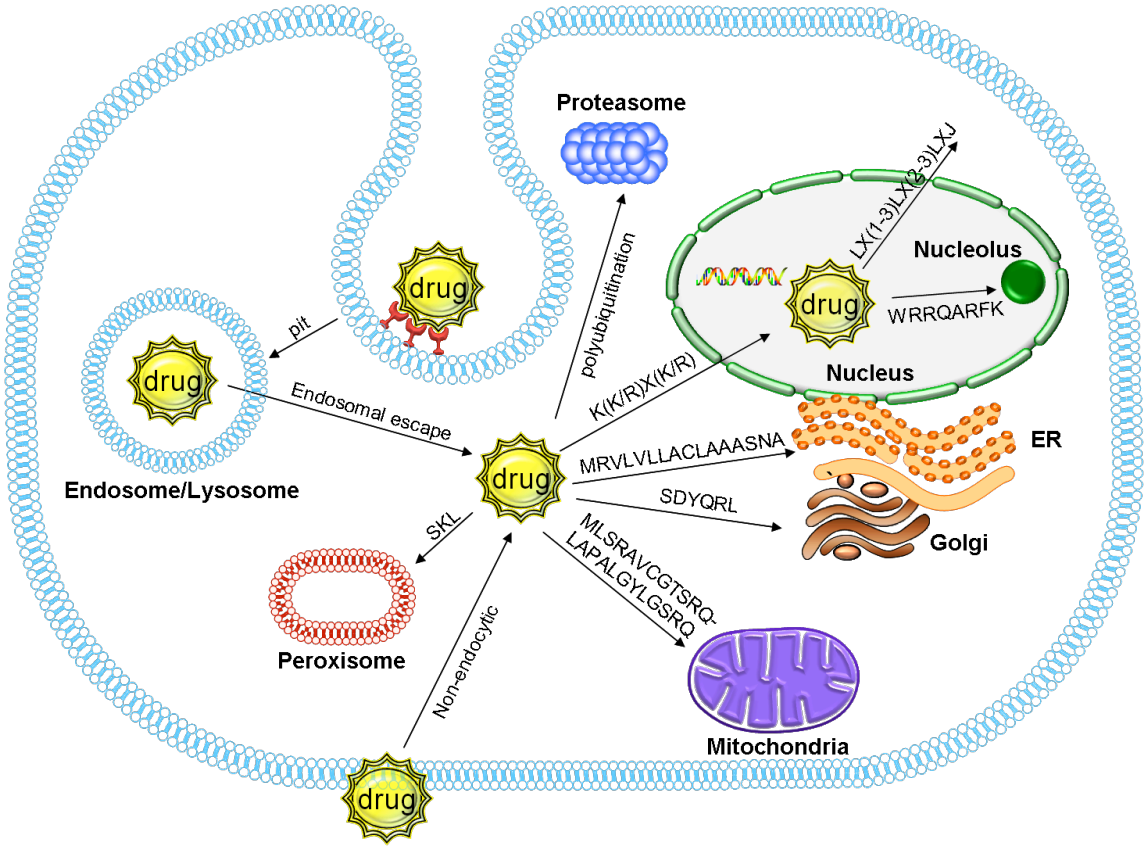


Figure 2.1. Drug targeting to cellular organelles. Text on arrows indicate consensus targeting sequence (if available), example of targeting signal, or mode of targeting.

to an alteration in function. Ultimately we seek not only targeting of a specific organelle but also a further level of sophistication, where multiple signals can be used to target multiple organelles. Indeed, the ability to precisely target drugs to different organelles is changing the way therapeutics are developed. This paper will focus on therapeutic targeting to specific cellular organelles (Figure 2.1). Each organelle will be briefly described, followed by methods to reach and target the organelle for therapy. Finally, future perspectives for therapeutic delivery will be discussed.

Cytosolic Delivery

Typically, drugs targeting cellular organelles have to be initially delivered to the cytosol, which in some cases is the site of action, where drugs (such as glucocorticoids, proteins, or siRNA) bind to their receptors or act on other targets. There are three main barriers, which must be overcome to facilitate cytosolic delivery: i.) evading detection by the reticuloendothelial system (RES), ii). interaction with the cell membrane and internalization; and iii.) intracellular trafficking and endosomal escape.

Evading RES Detection

In order for cytosolic delivery to occur when the drug is delivered into the bloodstream, the drug first has to evade detection by the RES prior to interacting with cell membranes. The endothelial layers of the liver, spleen and bone marrow comprising the RES contain mononuclear macrophages that filter the blood of foreign pathogenic particles (*8*). Aggregation of foreign particles in the presence of plasma proteins and cell adhesion molecules facilitates rapid clearance from the bloodstream (*9-12*). To lengthen

the circulatory time and decrease macrophage detection, several approaches have been attempted. Liposomes have been one of the most common drug delivery agents used to evade RES detection. Regular liposomes undergo rapid opsonization via the RES cells, followed by lysosomal degradation. Strategies to evade RES detection have included use of targeted liposomes (for accumulation in target organs) and or using "stealth" liposomes [21]. Long circulating liposomes (stealth) can be prepared by including amphiphilic stabilizers (e.g., cholesterol) (*13-15*), phosphatidylinositol and gangliosides (*16*), or a hydrophilic surface by grafting with polyethylene glycol (PEG) (*17*). The combination of long-circulating and targeted liposomes has been extremely popular in the last decade (such as antibody targeting and PEG) [21].

Cell Membrane Interaction

Subsequently, therapeutics interact with cell membranes (plasma membranes), which are lipid bilayers composed of phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin are common phospholipids), cholesterol, glycolipids, and proteins (including proteoglycans, which contain a core protein with 1 or more glycosaminoglycans attached) (*18*). Proteoglycans are important for delivery of drugs via cationic polymers (*19, 20*). For example, an ionic interaction occurs between cationic polyethyleneimine (PEI), and the cell membrane which has negatively charged sulfated proteoglycans on the cell surface (*19*).

Conjugation with cell penetrating peptides (CPPs) can also overcome the barrier of the cell membrane (*21, 22*). In the late 80s and early 90s many groups ((*23-25*) and others) found that a protein transduction domain of 9-16 cationic (polyarginine like)

amino acid residues had cell membrane-penetrating capability, including the ability to cross the nucleus. Several other groups (*26*) showed that peptides and proteins attached to CPPs can cross cell membranes (reviewed in (*27*)). Many therapeutics have been delivered to cells in this manner (*28-32*).

Internalization may occur by endocytosis (initiated by electrostatic or hydrophobic interactions with the cell membrane, or interaction with a cell surface receptor) followed by endosomal escape, or by other means, such as macropinocytosis, or combinations of these (*33*). After trafficking into the cytosol, the drug may either exert its action or traverse to a subcellular compartment (nucleus, mitochondria, peroxisome, etc.). For example, for delivery of genes, the cytoplasmic barrier is only the first step before delivery to the nucleus can occur. Table 2.1 depicts various agents designed to target the cytosol and their corresponding cargo (drug).

Liposomes, cationic lipid DNA and polymer complexes have been used as non-viral delivery carriers by complexing with DNA (*34, 35*). Nanoparticles can encapsulate various types of therapeutics including low molecular weight drugs (*36, 37*), and macromolecules (e.g., proteins and DNA). Liposomes bind to cell membranes and are internalized via endocytosis with pH-sensitive liposomes being more efficient (*38, 39*). The formulation of pH-sensitive liposomes with phosphatidylethanolamine (PE) increases affinity to adhere to cell membranes due to aggregate formation because of the poor hydration of its head-groups (*40, 41*). Other liposome examples include thermosensitive liposomes, which allow temperature-sensitive release of drug (*42-50*). Development of drug carriers is still in progress to overcome cytosolic barriers, including biodegradable polymeric carriers for controlled release (*51, 52*).

Table 2.1. Cytosolic targeting of drug therapeutics (includes endosomal/lysosomal escape, in some cases).

Class of targeting agent	**Targeting agent composition**	**Drug delivered (trade name if available)**	**References**
Liposomes	Liposome composed of lipid bilayer neutral lipids, DPPC and cholesterol	Amikacin (Arikace™)	(*53*)
	Liposomal includes soybean oil and phospholipids	Cyclosporin	(*54*)
	Doxorubicin STEALTH® liposome containing MPEG-DSPE, HSPC and cholesterol	Doxorubicin (Doxil™)	(*55*)
pH sensitive liposomes	Liposomes composed of CHEMS and DOPE	Diptheria toxin A chain	(*38*)
	DOPE, *N*-succinyl-DOPE, and PEG-ceramide	Gentamycin	(*56*)
Thermosensitive liposomes	DPPC, HSPC, and cholesterol liposomes surface modified with DSPE-PEG-2000: PNIPAM-AAM17	Doxorubicin	(*57*)
Targeted thermal magnetic liposomes	DPPC, cholesterol, DSPE-PEG(2000), DSPE-PEG(2000)-Folate)	Doxorubicin	(*58, 59*)
Polymeric micelles	Poly(ethylene glycol)-poly(aspartic acid) block copolymer	Doxorubicin (Adriamycin®)	(*60*)
pH sensitive micelles	Poly(ethylene glycol)-block-poly(aspartate-hydrazide) or PEG-p(Asp-Hyd) was modified using either levulinic acid (LEV) or 4-acetyl benzoic acid (4AB) attached via hydrazone bonds	Doxorubicin (Adriamycin®)	(*61*)
pH sensitive micelles with cell surface targeting	amphiphilic block copolymers that self-assemble into spherical micelles, folate-poly(ethylene glycol)-poly(aspartate-hydrazone-adriamycin) with gamma-carboxylic acid activated folate	Doxorubicin (Adriamycin®)	(*62*)

Table 2.1 Cont.

Thermosensitive micelles/polymers	micellar cyclotriphosphazenes	Human growth hormone	(*43*)
	Biodegradable triblock copolymer of PLGA-PEG-PLGA (ReGel®)	Paclitaxel	(*63*)
Cell penetrating peptides	Doxorubicin bound to HPMA-based polymer with the cell penetrating peptide Tat	Doxorubicin	(*64*)
Cationic polymers and cationic lipids	Polyethyleneimine	Genes (DNA)	(*65, 66*)
Virus-mimetic	Hydrophobic polymer core poly(L-histidine-co-phenylalanine)(poly(His_{32}-co-Phe_6)and 2 layers of hydrophilic shell (one PEG end linked to core polymer; other end to BSA)	Doxorubicin	(*67*)
Nuclear Export Signal (NES)	NES (LQLPPLERLTL) encoded in a plasmid	Genes (DNA)	(*6, 7, 68*)
	NES (ALPPLERLTL) conjugated to DNA	Antisense oligonucleotide	(*69*)

Conjugating antibodies to either liposomes or polymers increases internalization via a receptor-mediated endocytotic process (*70, 71*). Others have targeted ligands to their respective cell-surface receptors (folate receptor (*72*), transferrin receptor (*73*), LDL receptor (*74*) and many other ligand-receptor mediated methods (*75*)) for endocytosis and eventual release into the cytoplasm.

It may be desirable to retain a protein or peptide, once inside a cell, in the cytoplasm, where it may interact with its target (such as a signal transduction protein or receptor). Proteins smaller than about 45kDa can passively diffuse into the nucleus; exclusion from the nucleus (hence cytoplasmic localization) can also be conferred by increasing the size of the gene therapy protein to over 60kDa (*76*). Such large proteins cannot traverse the nuclear pore complex (NPC) passively, and require nuclear localization signals for entry into the nucleus via the NPC. Proteins, peptides and DNA may be tagged with amino acid sequences that confer nuclear export (using a nuclear export signal, or NES) to achieve this goal, or in the case of a gene, the NES may be encoded by plasmid DNA and genetically engineered to the therapeutic gene. Our laboratory uses the latter to confer cytoplasmic localization to engineered proteins (see Table 2.1) (*5-7, 68*). NESs are composed of sequences that are leucine rich, approximately 10-12 amino acids in length. Proteins with NESs are exported out of the nucleus by the classical export receptor, CRM-1 or exportin-1 (*76*) (Figure 2.2A). A common consensus NES is LX (1-3) LX (2-3) LXJ where L is leucine, X is a spacer (numbers in parenthesis indicate number of amino acids in the spacer) and J is leucine, valine or isoleucine (*6, 7, 68*).

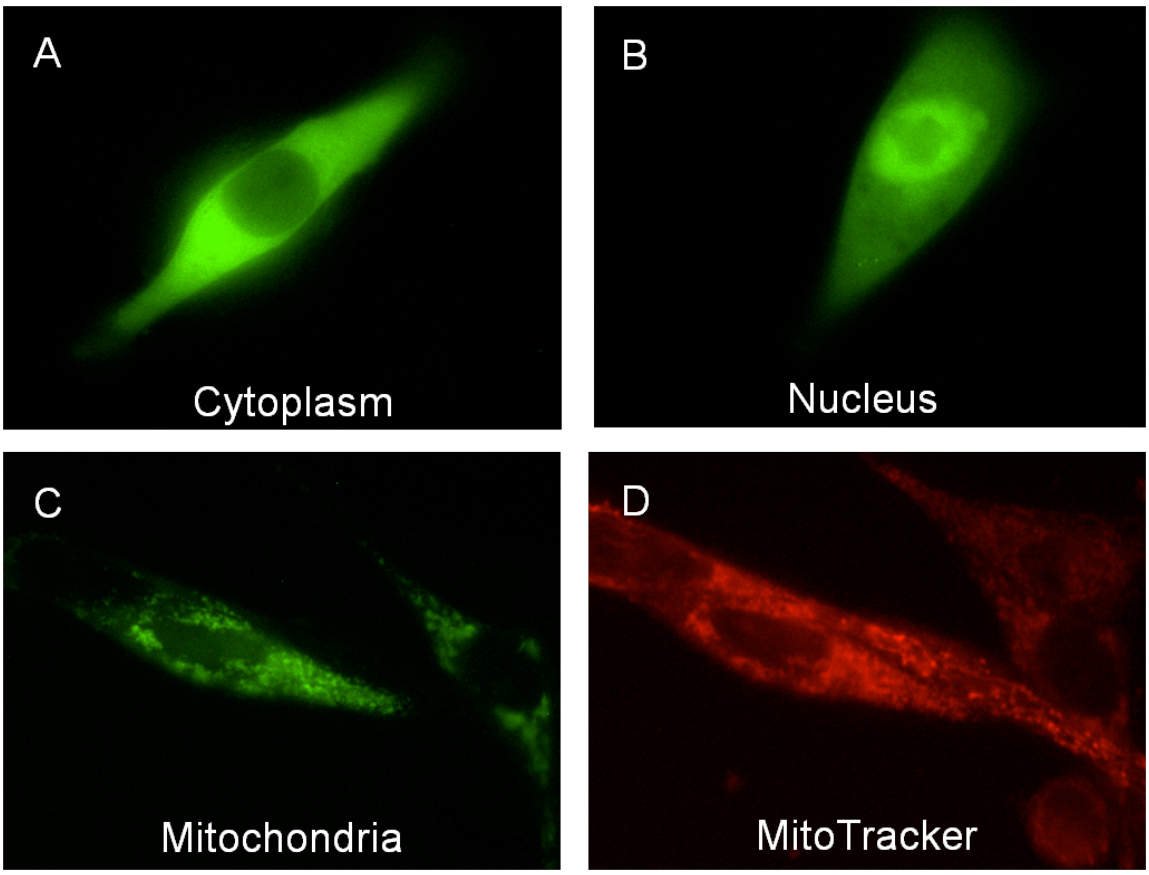


Figure 2.2. EGFP fused to different targeting signals and transfected into 1471.1 murine adenocarcinoma cells. A) Nuclear export signal (HIV NES) delivers EGFP to cytoplasm. B) Nuclear localization signal (MycA8 NLS) delivers EGFP to nucleus. C) Mitochondrial targeting signal (ornithine transcarbamylase MTS) delivers EGFP to mitochondria. D) MitoTracker (Invitrogen) stains mitochondria in live cells.

The Endosome/Lysosome

Endosomal/Lysosomal Barriers

Drugs that have entered cells via endocytosis (and need to be delivered to the cytosol) are faced with a third obstacle: late endosomal/lysosomal degradation. The process of endocytosis starts at the cell surface. The material to be internalized gets encapsulated by a small portion of the plasma membrane (clathrin coated pit formation), followed by a pinching off to form an endocytic vesicle. In the case of pinocytosis, some vesicles may originate at caveolae (instead of clathrin). Receptor-mediated endocytosis is a common mechanism for endocytosis of drugs/drug carriers. Endocytosed material that is not retrieved from endosomes (in the case of recycled receptors, for example) is destined for the lysosome (*18*). Lysosomes contain hydrolytic enzymes for digestion of macromolecules derived intra- or extracellularly; for digestion of phagocytosed materials, and even for production of nutrients (*18*).

The classic example uses polyethyleneimine (PEI) for endosomal escape (*65*). PEI's secondary and tertiary amines are protonated in the acidic environment of the endosomes. The "proton sponge effect" causes osmotic swelling and rupture of the endosomes (*77*). The main drawback of PEI is its toxicity (*78, 79*), but many modifications of PEI have been made to attempt to circumvent this (*80-84*).

Many other techniques to escape the endosome (hence bypassing degradation) have been investigated. These include the development of pH-sensitive liposomes and polymers, where they switch from a membrane inert (pH 7.4) to a membrane disruptive (under acidic condition) conformation (*85*). This facilitates a destabilization of the endosomal membranes, resulting in release of the encapsulated therapeutic in the

cytoplasm (reviewed in (*37*)). Further, membrane-disruptive polymers masked by PEG via disulfide groups and acid-degradable acetal groups disrupt the endosomal membrane when the acidic pH of the endosome degrades the acetal groups unmasking the membrane-disruptive backbone (*86*). Subsequently, the disulfide groups are reduced in the cytoplasm releasing the conjugated drug. Another strategy is to use membrane disruptive peptides mimicking the viral mechanism of endosomal escape. These peptides contain acidic residues that prevent the formation of an α-helix when unprotonated, but under slightly acidic pH the peptide forms an α-helix which allows multimerization and protein interaction leading to endosomal escape.

Endosomal/Lysosomal Targeting

A drug may also be targeted to the endosome/lysosome directly for a therapeutic effect. There are over 40 lysosomal storage diseases that occur as a result of lysosomal enzyme deficiency which allow various proteins, lipids, and carbohydrates to accumulate in the lysosomes. These diseases manifest usually as neurodegenerative, but can be treated with enzyme replacement therapy (reviewed in (*87*)). Drug delivery to endosomes has been achieved by targeting cell surface receptors (mannose 6-receptor, reviewed in (*88*), the IGF-II/cation-independent mannose 6-phosphate receptor (*89*)) for receptor mediated endocytosis.

Nucleus

Transport Through the Nuclear Pore Complex

Nucleocytoplasmic exchange occurs via pores formed by nuclear pore complexes embedded in the nuclear envelope (*90*). The 125 MDa nuclear pore complex (NPC) is the gatekeeper for nuclear entry, and the central role of this complex is confirmed by the high level of conservation among all eukaryotes (*91-93*). This complex regulates all trafficking into and out of the nucleus, including passive diffusion of molecules smaller than 9 nm in diameter (<40 kDa) and active transport of molecules between 9-40 nm in diameter (40 kDa to 60 MDa), and has an estimated ability to translocate 1000 molecules per second (*94-96*). Cytoplasmic filaments, a central channel, and a nuclear basket give the NPC complex a tripartite structure constructed from approximately 100 nucleoporins (Nups) (*97-99*) (reviewed in (*5*)). The long cytoplasmic filaments project into the cytoplasm and interact with karyopherins (family of transport receptors) and deflect non-shuttling proteins (*100-103*). The central channel is a cylindrical intramembrane transporter (central aqueous channel) with a 9 nm diameter pore (*104-106*), and is anchored to the nuclear membrane via eight spokes. The nuclear basket consists of eight long filaments projecting into the nucleoplasm, which are connected distally by a ring.

While molecules that are small enough to fit through the 9 nm pore in the central channel can enter the nucleus passively, larger molecules must be escorted through the NPC complex by karyopherins. Karyopherins, such as importin α, recognize and bind specific amino acid sequences termed nuclear localization signals (NLS) (*76, 107-110*). Classical NLSs are monopartite with a single stretch of amino acids similar to the NLS found in SV-40 large tumor antigen (PKKKRKV; critical residues underlined) (*111*), or a

consensus of K(R/K)X(R/K) (*112*) (Figure 2.2B). Bipartite NLSs (two short amino acid sequences with a spacer in between) have also been identified in proteins like nucleoplasmin (KRPAATKKAGQAKKKKLDK) (*113*). Classical NLS sequences are recognized by importin α (*114, 115*), which binds to the HEAT repeats (helical repeats of histidine, glutamic acid, alanine, and threonine) of importin β (*116*) through an importin β binding (IBB) domain (*117*). Once this ternery complex is formed, importin β mediates the translocation of the complex into the nucleus through interactions with FG repeats of Nups in the NPC (*118*). Inside the nucleus the complex dissociates when RanGTP binds to importin β (*119*), and both importin α and β are escorted separately back to the cytoplasm by RanGTP (*115, 120, 121*). In the cytoplasm, RanGAP catalyzes the conversion of RanGTP to RanGDP, and importin α and importin β are ready for another cycle of import.

Challenges of Nuclear Trafficking

It is not a simple task to diffuse through the highly viscous cytosolic fluid. DNA over 2 kb is almost immobile, and DNA over 250 bp has reduced lateral mobility (*122, 123*). Nevertheless, a mechanism to overcome the challenge of diffusing through the cytosol is demonstrated by viruses, which use active transport along the microtubule cytoskeleton (*124-127*). Another challenge in nuclear targeting is the translocation through the nuclear envelope, which has been described as one of the limiting steps for non-viral gene delivery (*128*). The midplane of the pore in the NPC is the narrowest, and is estimated to be approximately 40 nm in diameter (*129*). Thus, the largest molecule able to fit through this pore and enter the nucleus is roughly 40 nm, an estimate that has

been confirmed (39 nm) using gold nanoparticles coated with NLSs (*130*). The 36 nm capsid from hepatitis B can also transport through the NPC into the nucleus without dissociating (*130*), further demonstrating the capabilities of the NPC and validating the 40 nm size limitation. However, plasmid DNA in complex with polylysine crosslinked with NLSs, 60 nm in diameter, has been shown to enter the nucleus (*131, 132*), likely due to the increased flexibility of this complex versus the more rigid gold nanoparticles. Molecules larger than 40-60 nm in diameter, regardless of containing a NLS, will be unable to enter the nucleus (*131, 132*).

Methods of NLS Incorporation

Various methods of incorporating the NLS into the therapeutic have been attempted (Reviewed in(*128, 133*)). For peptide/protein therapeutics, genetic engineering provides a facile method of incorporating the NLS as we have recently demonstrated with the oncoprotein Bcr-Abl which causes chronic myelogenous leukemia (CML) (*4*). However, for nonpeptide/protein therapeutics, the attachment of the NLS is no trivial matter, and can result in drastically different results. As gene therapy is one of the most extensively researched areas in nuclear targeting, Table 2.2 provides examples of various methods of linking NLSs to DNA for gene therapy.

Of these approaches, electrostatic interactions are the simplest, but are not without drawbacks. One potential problem is the dissociation of the complex in the cytosol, leaving the DNA without the nuclear targeting signal. Furthermore, as most electrostatic interactions with DNA are nonsequence specific, the interactions may interfere with the

Table 2.2. Methods to link NLSs (Nuclear Localization Signals) to DNA-based therapeutic agents.

Type of Association	**Description**	**Reference**
Electrostatic	NLS in complex with DNA	(*134-138*)
Electrostatic	NLS-PLL in complex with DNA	(*139*)
Electrostatic	M9-ScT in complex with DNA	(*140*)
Electrostatic	NLS-PNA in complex with DNA	(*141-145*)
Electrostatic	Triplex formation between Padlock Oligonucleotide with NLS and DNA	(*146*)
Electrostatic	NLS-Streptavidin bound to Biotinylated DNA	(*147*)
Covalent (Random)	N3-adenine adduct via cyclopropapyrroloindole (CPI)-NLS	(*148*)
Covalent (Random)	Photoactivation of p-azido-tetrafluoro-benzyl-NLS	(*149*)
Covalent (Random)	Diazo coupling to increasing lengths of PEG-NLS	(*150*)
Covalent (Specific)	Cross-linked triple helix with psoralen-oligonucleotide	(*151*)
Covalent (Specific)	Attachment to hairpin of linear DNA	(*152-154*)
Covalent (Specific)	Attachment to PCR primer	(*154, 155*)

transcription of the gene after being delivered to the nucleus. To circumvent this problem, peptide nucleic acid (PNA) with a NLS has been used to interact with the DNA in a sequence specific manner in a region other than the gene of interest (*141*), but only achieved modest improvements. Numerous attempts to improve efficiency through covalent attachment of the NLS have been attempted, but many have failed to demonstrate improved nuclear translocation and gene expression (*148-150, 156*). However, initial attempts to covalently attach the NLS to the DNA did not control where on the DNA strand the NLS was attached and may have resulted in the NLS preventing the efficient transcription of the gene of interest.

Further attempts were to then attach the NLS to DNA upstream or downstream from the gene of interest so as to avoid interference with transcription. Nevertheless, in spite of various attempts to conjugate the carrier to the DNA away from the gene of interest, this approach has not demonstrated the anticipated results. The triple-helix with photoactivation attempted by Neves et al. (*151*), the conjugation to the hairpin attempted by Tanimoto et al. (*153*), and linear DNA amplified via PCR with NLS-conjugated primers (*154, 155*) all demonstrated a lack of transfection efficiency, and even a lack of nuclear tanslocation. However, one study has shown that covalent attachment of a NLS to a hairpin at the 3'-end of linear DNA can enhance transfection anywhere from 10 to 1000 fold depending on the cell type (*152*), but similar schemes did not achieve the same result by others.

Therapeutics Targeting the Nucleus

Pharmacological agents that alter the nuclear translocation (either inhibit or induce translocation) also provide a means for therapeutic intervention (*157*). Inhibition of the NPC through binding FG repeats by antibodies has been used to prevent nuclear accumulation (*158*) but is not specific and inhibits all protein translocation into the nucleus. These antibodies, RL2 and mAb414, are further limited by complications with delivery of an antibody inside a cell. Wheat germ agglutinin (WGA) is a lectin that binds to N-acetylglucosamine common to certain Nups and also inhibits general nuclear transport through the NPC (*159, 160*). Kosugi et al. have also demonstrated that nuclear translocation can be inhibited through two peptides, bimax1 and bimax2, that bind to importin α (*161*). The inhibition of the NPC reduces nuclear transport and results in reduced nuclear localization, while the inhibition of nuclear export can increase the nuclear accumulation of proteins. Leptomycin B (LMB) is a general nuclear export inhibitor that binds to Crm1 (*162-164*), a protein that escorts other proteins with nuclear export signals (NESs) from the nucleus to the cytoplasm. Although LMB has antitumor properties, it was also found to be highly toxic and cannot be used clinically. Recently, Mutka et al. have identified LMB analogs that retain the potency of LMB, are better tolerated, and have shown potential as cancer therapeutics in mouse xenograft models (*165*). Still, inhibiting the general nuclear import or export of all proteins may be limited as a therapeutic approach and methods to target specific proteins would be a better alternative.

An example of therapeutic intervention through altering the nuclear localization of a specific protein is the use of peptides that bind nuclear factor-κB (NF-κB) and

prevent its nuclear translocation (*166, 167*). The misregulation of the transcription factor NF-κB has been associated with cancer and autoimmune diseases, both of which potentially could be treated by inhibiting the nuclear accumulation of NF-κB (*168*). A similar approach of inhibiting nuclear translocation through binding with a peptide has been demonstrated for nuclear factor of activated T cells (*169*). Moving proteins from the cytoplasm into the nucleus has also been demonstrated through the use of protein aptamers containing a NLS (*170*). We have also developed a sophisticated chimeric protein we have termed a protein switch (Figure 2.3), for controlled nuclear transport (*6, 7, 68*). This protein switch (containing both NES and inducible NLS) localizes to the cytoplasm, but upon the addition of a ligand is translocated into the nucleus. This controlled nuclear translocation can be used to alter the nuclear localization of endogenous proteins, and attempts to do so are currently underway in our laboratory.

Targeting the Nucleolus

A subcellular compartment such as the nucleolus presents additional challenges in identifying specific targeting sequences as it exists in a highly dynamic equilibrium with the nucleoplasm and is not enclosed by a membrane. The nucleolus has been described as a machine for ribosome production, and is a dense area composed of nucleolar organizer regions (loops of DNA containing genes encoding rRNA), rRNA, ribosomal proteins, ribosomal-binding proteins, small ribonucleoprotein particles (snRNPs), and RNA polymerase I. The nucleolus typically exhibits fibrillar centers, fibrillar components, and granular components. The fibrillar centers are regions where

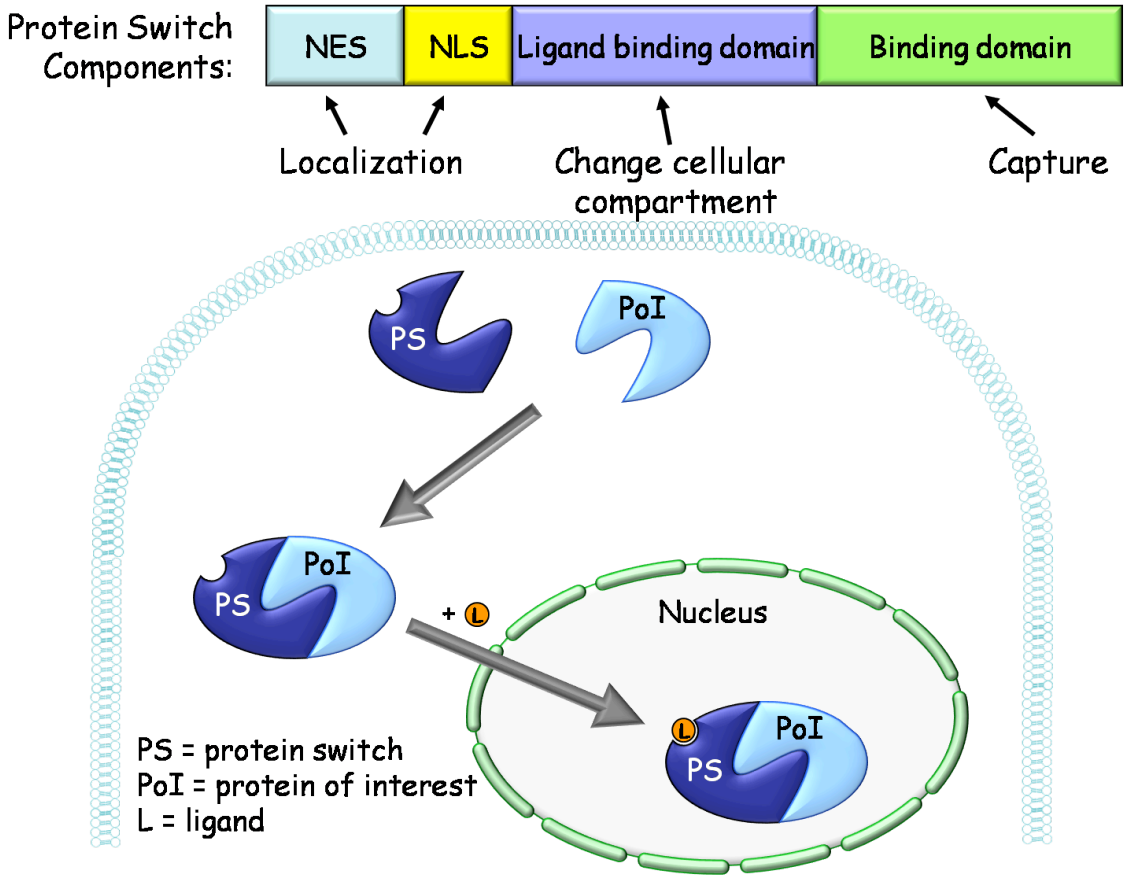


Figure 2.3. Protein switch mechanism. The PS, shown schematically (top diagram), consists of a NES, NLS, ligand-binding domain and dimerization domain. When PS plasmid is transfected into cells, the expressed PS captures a poI in the cytoplasm. Upon addition of ligand, the PS-poI complex translocates to the nucleus.

L: Ligand; NES: Nuclear export signal; NLS: Nuclear localization signal; PoI: Protein of interest; PS: Protein switch.

transcription is not taking place, where as the fibrillar components are more dense regions where transcription is underway, and the granular components are regions where the ribosomal precursor particles are undergoing maturation.

Various sequences (reviewed in (*171*)) with as few as seven amino acids (*172*) have been identified that can be used to direct a protein to the nucleolus. The nucleolus localization signals (NoLSs) are very similar to the arginine/lysine rich NLS sequences, which is easy to conceive due to the necessity to first be transported into the nucleus before targeting the nucleolus. However, there is a distinction between NLSs and NoLSs as demonstrated through deletion analysis of the NoLS in the ORF57 protein (among others) resulting in diminished nucleolar localization while retaining nuclear localization (*173*). Through alignment of various proteins containing NoLSs, Weber et al. (*174*) identified a common NoLS motif (R/K)(R/K)X(R/K). This highly conserved sequence was also noted by Horke et al. after finding it to be important for nucleolar localization in the human La protein (*175*). Although there is still not a consensus NoLS, and the nucleolar targeting is not as well characterized as other signals, the addition of short NoLS sequences have been demonstrated to direct proteins to the nucleolus (*172, 176*), validating their use in targeting the nucleolus.

Proteins without an identifiable NoLS have been shown to localize to the nucleolus via interactions with other molecules at the nucleolus. One such example is nucleolin, a protein which may localize to the nucleolus partially due to RNA binding. However, the RNA binding domains are not sufficient to cause nucleolar localization of chimeric proteins (*177*). Additionally, NoLS-containing proteins, such as NPM (*178*) and NOM1 (*179*), have been shown to bind and cause a nucleolar localization of proteins

that do not contain a NoLS, further expanding the mechanisms for nucleolar localization. Emmott and Hiscox (*171*) have recently proposed that nucleolar hub proteins play an essential role in nucleolar localization. Thus, nucleolar targeting can be achieved through NoLSs, by being dragged by a NoLS-containing protein, or potentially by binding to a nucleolar hub protein.

There are relatively few drugs that target the nucleolus or nucleolar components specifically, but actinomycin D has been shown to induce nucleolar segregation due to the inhibition of rDNA transcription (*180, 181*). Phosphorylation is a critical modification in the regulation of nucleolar proteins, and kinase inhibitors have drastic effects on the nucleolus (*182-184*). One such inhibitor, the casein kinase inhibitor 5,6-dichloro-1-ribo-furanosylbenzimidazole (DRB), causes the nucleolus to disassemble (*185*). An example of a more specific interaction involving a potential nucleolar targeting therapeutic is the peptide consisting of amino acids 26-46 of the $p19^{ARF}$ protein (*186*). This peptide, modified with additional arginine residues to increase cellular uptake, has been shown to bind Foxm1b and send it to the nucleolus where it is unable to function as a transcription factor. Foxm1b has been shown to have a key role in the development of hepatocellular carcinoma (*187*), and the nucleolar sequestration of Foxm1b presents a novel therapeutic intervention.

Mitochondria

Mitochondria contain a double membrane composed of phospholipid bilayers with proteins embedded in them. This results in two aqueous spaces; the matrix and the intermembrane space. The inner membrane contains the proteins involved in the

respiratory chain complex, ATP synthase and protein import machinery. The human mitochondrial matrix encloses a small genome (mtDNA) coding for 13 hydrophobic proteins, 22 tRNAs and two rRNAs (*188, 189*). The 13 hydrophobic proteins encoded in the matrix are all involved in the electron transfer system. The matrix is also the site for the citric acid cycle, urea cycle and fatty acid oxidation. Therefore, mitochondrial dysfunction contributes to a range of human diseases including obesity, diabetes and cancer. Mitochondrial targeting is mainly performed to interrupt mitochondrial function including energy production, oxidative stress and the apoptotic pathway.

Mitochondrial Function

The mitochondria maintain an acidic inner space and an electrochemical potential across the inner membrane, which are utilized by the electron transport system to transfer electrons to oxygen. The electron transport chain passes electrons from the reducing equivalents to oxygen. During this process, protons are pumped from the matrix to the intermembrane space via NADH dehydrogenase, cytochrome c reductase, and cytochrome c oxidase. As a result, an electrochemical gradient is established, which activates ATP synthase to pump protons back in and activates ADP to ATP as the energy source for the cell (*190*).

In the mitochondrial respiratory chain, oxygen is partially reduced to form reactive oxygen species (ROS) activating the proton leak uncoupling proteins, which pump protons back into the matrix without forming ATP (*191-193*). Superoxides are also converted to other ROS such as hydroxyl radicals, which contribute to a number of degenerative diseases (*194-198*). The oxidative damage, which increases with ageing,

also affects lipids, proteins and DNA leading to a decline in the efficiency of oxidative phosporylation (*195, 199*). Mitochondria destroy the free radicals using antioxidants (e.g., vitamin E, ascorbate, ubiquinol), which scavenge ROS, converting them to less reactive species or prevent oxidative damage. Therefore, antioxidants have been used as supplements to specifically target the mitochondria (reviewed in (*200*)).

Apoptosis is frequently triggered through the mitochondria after collapse of the inner transmembrane potential, disruption of electron transport and ATP production, oxidative stress, permeability transition pore opening, or mitochondrial swelling with outer membrane rupture. When the outer mitochondrial membrane undergoes permeabilization, cytochrome c is released from the intermembranous space into the cytosol activating caspase-3 via the Apaf-1 pathway (*201-203*). Mitochondrial outer membrane permeabilization is commonly disabled in cancer cells and hence its pharmacological induction constitutes a therapeutic goal (*204*). Bcl-2 and Bcl-XL are mitochondrial proteins that inhibit apoptosis by inhibiting Bax and Bak oligomerization (*205-209*).

Mitochondrial Import Machinery

Drugs targeting mitochondria are necessary to induce apoptosis in cancer cells as well as to protect cells from oxidative damage and to repair defects. The outer membrane of the mitochondria allows diffusion of small molecules through pores formed by spanning beta barrel protein called porin. Simple ions with localized charge such as Cl^{+}, Na^{+} or Ca^{2+} require translocators or energy-dependent transporters to allow them to cross into the matrix (reviewed in (*210*)). However, ions with delocalized charge and cationic

hydrophobic molecules such as triphenylphosphonium (TPP) can diffuse across the mitochondrial membrane taking advantage of the hydrophobic nature of the membrane (*211-213*). Conjugating antioxidants to TPP or a methyl derivative (TPMP) increases their accumulation within the mitochondria and selectively blocks mitochondrial oxidative damage (Table 2.3). TPP cation have been covalently attached to antioxidants such as ubiquinol (MitoQ) (*214-216*) and α-tocopherol (MitoE) (*217*). The same concept has been utilized to target DNA to the mitochondria using dequalinium, a delocalized cationic lipid (*218*). It crosses the plasma membrane by endocytosis, and then DNA is released upon interacting with the mitochondrial membrane (*219*).

Large molecules (e.g., proteins) exploit the mitochondrial protein import apparatus to cross the outer and inner membrane. The TOM complex (translocase of the outer membrane of mitochondria) is the molecular machine responsible for translocating proteins across the mitochondrial outer membrane. The TOM complex contains receptor subunits, Tom70 and Tom20, which recognize proteins destined for import (*220, 221*). Other TOM complex components, Tom40, Tom22, Tom7, Tom6, and Tom5, assist the transfer of the protein into the intermembrane space (*222-224*). The TIM complex (translocase of the inner membrane of mitochondria) binds to proteins destined for localization to the inner membrane and the matrix through TIM22 and TIM23, respectively (*222, 225*). The TIM22 complex translocates proteins depending on the electrochemical potential across the inner membrane. However, the TIM23 complex drives the protein trafficking via a motor complex built around a mitochondrial Hsp70 (*221*). Mitochondrial targeting signals (MTSs) are mainly N-terminal cleavable amino acids 15-40 residues in length, which are positively charged with a notable absence of

Table 2.3. Mitochondria targeting signals/motifs and delivered therapeutic agents.

Targeting Motif	**Therapeutic**	**References**
(2-oxo-ethyl)-triphenyl-phosphonium	Nitroxide (4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl)	(*226*)
Rhodamine B	Porphyrin	(*227*)
Stearyltriphenyl phosphonium (STPP)	Libosome-based nanocarrier containing ceramide	(*228*)
Triphenylphosphonium	Ubiquinol antioxidant	(*211*)
Dequalinium	DNA	(*218*)
MLSCTSPLLRGACHNMGAAKALRLR WTVPPAVLIALGSGALYTTSGQTLYY KNSVQQTD	Phospholipase A orthologue (AoPlaA)	(*229*)
MLSRAVCGTSRQLAPALGYLGSRQ	Protein transduction domain of HIV-1 TAT protein	(*230*)
MSATRMQLLSPRNVRLLSRGRSEL FAGGSGGGPRVRSLISPPLSSSSPG RALSSVSATRRGLPKEKMTENGV SSRAKVLT IDT	Alanine aminotransferase	(*231*)
MLFNLRILLNNAAFRNGHNFMVRN FRCGQPLQ	Tumor suppressor p53	(*232*)
MLFNLRILLNNAAFRNGHNFMVRN FRCGQPLQ	Dihydrofolate reductase (DHFR)	(*233*)

negatively charged residues (*234*). These MTSs forming amphipathic α-helices are thought to be important for their recognition by the translocation machineries in the mitochondrial outer (TOM complex) and inner (TIM complex) membranes (*221, 225, 235-238*). Once the protein is translocated into the matrix, the targeting signal is proteolytically removed by mitochondria processing peptidase (MPP) (*239, 240*). Nevertheless, a significant fraction of mitochondrial proteins, especially proteins of the outer membrane, the inner membrane space, and the inner membrane, lack typical N-terminal sequences and are targeted to the mitochondria by means of internal, noncleavable signals known as "carrier sequence motifs" (*241, 242*). These internal signals resemble the N-terminal sequence and are recognized by the TOM machinery (*243-245*). The internal signals function in combination with a preceding hydrophobic region (*246*).

Attaching MTSs to essentially any protein, DNA or RNA enables mitochondrial targeting (*232, 233, 247-249*) (Figure 2.2C and D). MTS has also been fused to restriction enzymes such as Sma1 endonuclease to degrade mutant mtDNA in NARP disease (*250*). Targeting proteins such as p53 to the mitochondria induces apoptosis by complexing with Bcl-XL proteins. The inactivation of Bcl-XL induces conformation change and oligomerization of the BH123 effector protein Bak and Bax, which then forms a pore in the outer membrane facilitating the release of cytochrome c (*251-253*). We are currently investigating the fusion of a strong NES to small proteins tagged with MTS to reduce nuclear localization and increase their availability in the cytoplasm to target the mitochondria (unpublished data). The effect of the different subcellular signals in a protein and the outcome of having multiple signals is a largely unexplored area, and

warrants attention. Table 2.3 lists examples of chemical (TPP) and biological (MTS) means to target therapeutics to the mitochondria.

Endoplasmic Reticulum

The endoplasmic reticulum (ER) is an organelle found in all eukaryotic cells. Its membrane represents as much as half of the total membrane of an average animal cell. It extends from the nuclear envelop to the cell periphery intertwining with most cellular organelles, including the mitochondria, peroxisome and the Golgi apparatus (*254*). Regions of the ER that are coated with ribosomes are called rough ER, where proteins are imported into the ER in a co-translational process. In co-translational transport, one end of the protein is translocated into the ER while the rest of the protein is being assembled in the ribosome. Smooth ER is the region that lacks bound ribosomes. An essential function of the ER is the biosynthesis of proteins and lipids that are destined for intracellular organelles and the cell surface (*255*). It also controls Ca^{2+} signaling via its Ca^{2+}-binding proteins and homeostasis (*256*).

ER Targeting

The co-translational targeting of proteins to the ER membrane (transmembrane proteins) and lumen (water soluble proteins) is mediated by a cytosolic ribonucleoprotein called the signal recognition particles (SRPs) and their cognate membrane-associated receptors (SRs) (*257-259*). One end of the SRP binds to the ER signal sequence as they emerge from ribosomes, while the other end of the SRP blocks the translational elongation in the ribosome halting protein synthesis (*260-263*). The signal sequence

consists of 7-12 large hydrophobic residues that presumably form an α-helix (*263-267*). The SRP-ribosome complex is then directed to the ER membrane by binding to the SRP receptor, which is an integral membrane protein complex embedded in the rough ER membrane (*258, 268-271*). This binding releases the ribosome complex to the integral membrane protein Sec61 translocation complex (translocon), allows the translational elongation to continue and recycles the SRP back to the cytosol (*272, 273*).

The ribosomal exit tunnel is aligned to a water filled pore in the translocon through which the protein is translocated and continues being translated. Subsequently, the signal sequence is cleaved via an ER signal peptidase and released into the membrane, where they get degraded by other proteases. The translocon also allows a translocating protein lateral access into the hydrophobic core of the membrane for both integration of membrane proteins into the bilayers (via either a stop transfer sequence or a signal anchored sequence) and the release of the cleaved signal peptide into the membrane (for review see (*274, 275*)). The ER is not limited to co-translational targeting of proteins; some post-translational proteins are imported into the ER in a SRP-independent mechanism, where they depend on the hydrolysis of ATP and the binding to *cis*-acting molecular chaperones such as heat shock cognate protein 70 (Hsp70) (*276-281*). The signal sequences in this category are no more than 70 amino acid residues in length (*279, 282*).

The ER signal sequences have substrate-specific differences, which may have functional consequences (*283, 284*). The signal sequences may differ in their gating of the translocation complex (*285, 286*), dependency on the translocon-associated proteins (*287, 288*), translocation efficiency of proteins (*289-291*), or sensitivity to translocation

inhibitors (*292, 293*). Table 2.4 lists examples of ER signal sequences fused to therapeutic proteins.

ER Function

Nearly one third of the eukaryotic genome is processed through the ER (*294*). The transferred transmembrane proteins either function in the ER or reside in the membrane of other organelles or the plasma membrane. Similarly, the water-soluble proteins are either secreted to the cytosol or transferred into the lumen of other organelles. Transmembrane proteins directed to the ER include plasma and organelle membrane receptors and channels that regulate metabolic pathways, regulate cell to cell communication and flow of ions and solutes and mediate protein and lipid uptake from the surrounding cellular environment. In addition, secreted proteins and enzymes are required for cell communication with its surrounding and to support sorting, metabolic and catabolic activities (For review see (*295*)). Therefore, a wide range of diseases occur due to mistakes in protein handling in the ER such as cystic fibrosis, liver failure, Alzheimer, Parkinson and diabetes mellitus (*296-304*) (For a list of diseases see (*295*)).

The Golgi Apparatus

The Golgi apparatus is known typically for posttranslational modifications and shipping macromolecules to the plasma membrane, lysosomes, or outside of the cell in secretory vesicles. Thus, the Golgi apparatus ships molecules in the opposite direction of drug delivery. Major players in this process, such as mannose 6-phosphate receptors (MPRs), are recycled back to the trans-Golgi network in a retrograde fashion (*305-307*)

Table 2.4. Endoplasmic reticulum targeting signals/motifs and delivered therapeutic agents.

Targeting Motif	**Therapeutic**	**References**
MDSKGSSQKAGSRLLLL LVVSNLLLCQGVVSTP	Mammalian prion protein (PrP)	(*308*)
ALAAALAAAAA	G-protein coupled receptors (GPCR)	(*309*)
MRVLVLLACLAAASNA	Recombinant proteins in baculovirus expression vector system	(*310*)
MLLPVPLLLGLLGLAAAL And KDEL (ER retention)	Human papilloma virus (HPV-16) E7 antigen	(*311*)
MRYMILGLLALAAVCSAA	Vesicular somatic virus nucleocapsid protein and influenza virus nucleoprotein	(*312, 313*)
MKFTVTFLLIICTLSAFC	Adenovirus type 5 E3 14.5-kilodalton protein	(*314*)
METDTLLLWV LLLWVPGSTGD	Transforming growth factor-β2 and –β3	(*315*)

demonstrating the potential for use in therapeutic targeting of the Golgi apparatus. However, this recycling pathway involves late endosomes (*305, 316, 317*) with the associated low pH and degradative enzymes that therapeutic delivery systems may want to avoid. However, retrograde delivery to the Golgi apparatus directly from early endosomes or recycling endosomes has been characterized with toxic proteins such as shiga and cholera toxins (*318, 319*). The receptor for these toxins is a glycolipid (globotriaosyl ceramide) that uses a pH-independent pathway (*319*) to target the Golgi apparatus that may have therapeutic potential. While clathrin is critical for efficient transport of shiga toxin (*320*), ricin has been shown to transport to the Golgi apparatus independent of clathrin and Rab9-GTPase (*321*), in a process regulated by cholesterol (*322*). Other proteins have also been shown to shuttle between the plasma membrane and the Golgi apparatus. One such example is a protein commonly used as a marker for the trans-Golgi network named TGN38/41, a receptor for p61 protein complexes and GTP-binding proteins, important in the formation of exocytic vesicles (*323*). Other examples are furin (*324*) and carboxypeptidase D (*325*).

The signaling sequences used by these proteins to target the Golgi apparatus are not well characterized and no specific consensus sequence has been proposed. However, tyrosine-containing motifs such as that found in TGN38/41 (SDYQRL) is one signal responsible for Golgi apparatus localization. Attachment of this signal to the LDL receptor has resulted in the localization to the Golgi apparatus (*326*). It has been shown that furin has two specific signals that target the Golgi apparatus: the acidic sequence CPSDSEEDEG, which is sufficient to cause localization to the Golgi apparatus, and the tyrosine-containing sequence YKGL, which has been proposed to function as a retrieval

signal through targeting of endosomes (*327, 328*). The localization of furin to the Golgi apparatus, and its shuttling to and from endosomes, is further dependent on phosphorylation by casein kinase II (CKII) at serine residues in the acidic cluster motif, and dephosphorylation by protein phosphatase 2A (PP2A) (*329, 330*), highlighting the role of posttranslational modifications on localization and not just specific amino acid sequences. A similar mechanism for targeting the Golgi apparatus via a tyrosine motif and a phosphorylation site has been demonstrated for the varicella-zoster virus glycoprotein I (VZV gpI) (*331*). While sequences targeting the Golgi apparatus have been studied and identified, little effort has been put forth to harness these signals for therapeutic delivery to the Golgi apparatus, leaving much to be explored.

Although the Golgi apparatus may not be the target of the therapeutic, delivery of therapeutics via the B subunit of Shiga toxin which enters the cell through the retrograde pathway involving the Golgi apparatus have been studied for vaccination, targeted killing of cancer cells, and imaging of cancer cells. Although the mechanism has not been delineated entirely, the MHC class I pathway involves the retrograde pathway through the Golgi apparatus, and Shiga toxin subunit B (STB) or Shiga-like toxin subunit B (SLTB) thus has been conjugated to various antigens for tumor protection (*332-336*). The receptor that binds ST/SLT is the globotriaosylceramide (Gb_3) found to be overexpressed on many tumor cells, allowing the retrograde pathway with ST/SLT or STB/SLTB to be used for tumor imaging (*337*) and targeted delivery of cytotoxic agents for colon (*338*), ovarian (*339*), and breast cancer (*340*), as well as lymphoma (*340, 341*), astrocytoma (*342*), and meningoma (*343*). Finally, since the Golgi apparatus has been described to exhibit a mechanism for stressed-induced initiation of apoptosis (*344*), this

validates future attempts at targeting therapeutics to the Golgi as a mechanism for specific induction of apoptosis in malignant cells.

Peroxisomes

The peroxisomes are multifunctional, single-membrane enclosed, spherical vesicles distributed throughout the cytoplasm (*345*). Their numbers vary in different cell types and under different stimuli. The term "peroxisome" was coined due to the fact that hydrogen peroxide is formed and degraded in the organelle. Peroxisomes are responsible for a wide variety of biochemical and metabolic pathways, with implications for human health. Aging and several diseases are associated with dysfunction of the peroxisomes, which has made it an interesting pharmaceutical target.

Peroxisomal Function

A major function of the peroxisome is the breakdown of a variety of fatty acids via α- and β-oxidation reactions. Similar to mitochondrial β-oxidation, fatty acids are broken down two carbons at a time in the form of acetyl-CoA. However, fatty acids containing a methyl group at the 3-carbon position must be modified before entering the peroxisomal β-oxidation pathway (*346*). Many of the metabolic pathways in the peroxisomes lead to hydrogen peroxide production, which is subsequently metabolized by the peroxidase catalase. Peroxisomes also play a role in the production of bile acids, docosahexanenoic acids and ether phospholipids.

Peroxisomal Import Machinery

The import machinery to the peroxisomal matrix requires (1) cargo containing specific targeting signals, (2) receptors that recognize specific signals, (3) membrane-associated import via docking and translocating proteins and (4) cargo release (reviewed in (*347*)). Cytosolic proteins require specific targeting signals recognized by receptors for peroxisomal targeting. Two types of peroxisomal targeting signals exist: type I (PTS1) and type 2 (PTS2). PTS1 is the most abundant, which consists of a tripeptide (SKL) or a conserved biochemical variant (consensus S/A/C-K/R/H-L/M) at the extreme C-terminus of the protein (*348-350*). An additional adjacent upstream region to the PTS also influences the interaction between receptor and PTS (*350-352*). In contrast, PTS2 consists of less conserved consensus sequence (R/K-L/I/V-X_5-Q/H-L/A, where X may be any amino acid) and is coded close to the N-terminus (*353-355*). For a list of bona fide peroxisomal proteins and their PTSs, review (*356*).

Specific proteins called receptor peroxins (Pex5p and Pex7p) bind to the proteins harboring PTS in the cytosol, which then target the peroxisomal membrane as a receptor/cargo complex. PTS1 is recognized by Pex5p while Pex7p recognizes PTS2 (*353, 357-359*). Pex5p is retained in the cytosol in the tetramer form. Upon binding to proteins containing PTS1, Pex5p disaggregates into dimers (*360*). Each dimer carrying two cargo proteins are then transported to the peroxisome. Subsequently, the receptor/cargo complex binds to membrane-associated peroxins (docking proteins), in particular Pex8p, Pex13p, Pex14p and Pex17p (*361*). The remaining membrane-associated peroxins (Pex2p, Pex10p and Pex12p) form the RING-finger subcomplex. Both the docking proteins and the RING-finger complex form the putative import

complex (importomer) which is thought to advance translocation (*362*). The precise mechanism for the peroxisomal protein import machinery remains ambiguous. The uniqueness in this mechanism is highlighted in its ability to transport folded, co-factor-bound and oligomeric proteins (*363*). There are two proposed models on the receptor's cycle between the cytosol and the peroxisome. The model of shuttling receptors states that the cargo dissociates from the receptor upon binding to the docking proteins (*353, 364*). The cargo is then translocated across the peroxisomal membrane while the receptor is released back to the cytosol. Alternatively, the extended shuttle hypothesis assumes that the dissociation between the cargo and receptor takes place in the peroxisomal matrix allowing the unloaded receptors to transport back to the cytosol (*365-367*).

Targeting the peroxisomal membrane (without translocating to the matrix) follows a different import mechanism. Peroxisomal membrane proteins (PMPs) require membrane protein targeting signals (mPTS). These signals contain a cluster of basic amino acids in conjunction with one or more transmembrane region downstream from it (*368-371*). The mPTS is recognized by Pex19p receptor in the cytosol, which then binds to Pex3p on the peroxisomal membrane. However, the exact mechanism on how PMPs are transported to the peroxisomal membrane is still unknown. There is also a possible but controversial involvement of the endoplasmic reticulum in PMP trafficking (*347, 372-374*). The importomer plays an important role for matrix targeting but not for membrane protein import.

Peroxisomal disorders are due to either mutations in peroxins (peroxisomal biogenesis disorder, PBD) or deficiencies in peroxisomal enzymes (reviewed in (*375*)). PBDs are categorized by means of clinical severity into the Zellweger spectrum of

disease [Zellweger syndrome, neonatal adrenoleukodystrophy (NALD) and infantile Refsum's disease (IRD)] or rhizomelic chondrodysplasia punctata (RCDP) type I. For example, a defect in the Pex7p disrupts enzymatic pathways for enzymes containing PTS2 causing RCDP type I. In addition to peroxins mutations that affect enzymes delivery to the peroxisome, single peroxisomal enzyme deficiencies have been linked to a number of human diseases such as adult Refsum's disease, X-linked adrenoleukodystrophy, RCDP type II and III, primary hyperoxaluria type I, acatalasemia, etc. (for a full list of diseases, see (*375*)).

Peroxisomal Targeting

Specific targeting to the peroxisome is of great clinical value to restore, alter or complement peroxisomal function. It could be used in the treatment of single enzyme deficiencies, PBD, as well as diseases associated with altered ROS (*375*). Tagging protein therapeutics or peroxisomal enzymes to a PTS is required to achieve peroxisomal targeting. The carboxy-terminal PTS1 (SKL or related sequence mentioned above) is better characterized and is easily tagged onto a protein of interest using standard recombinant molecular cloning techniques. The adjacent upstream region influences the receptor/PTS interaction and can be evaluated using a computer program (http://mendel.imp.ac.at/pts1/), which can also predict the relative strength of a PTS1. To be recognized by Pex5p, accessibility of the PTS1 (not buried in the folded protein) is also crucial. Therefore, a spacer between the PTS1 on the carboxy terminal and the protein could be introduced. Table 2.5 lists therapeutic proteins delivered to the peroxisome via PTS1.

Table 2.5. Peroxisomal targeting signals and therapeutics delivered.

Targeting Motif	Therapeutic	References
SKL	Catalase	(*376, 377*)
SKL	Monohydroascorbate reductase	(*378*)
SKL	Serine:pyruvate aminotransferase	(*379*)
AKL vs. NKF	Catalase	(*380*)

Proteasomal Function, Inhibition, and Potential Drug Delivery

The proteasome is an ATP-dependent protease that functions to remove (degrade) abnormal proteins found in the cytosol, nucleus, or ER. The entire proteasome (26S proteasome) consists of the 20S proteasomal core, capped by two 19S regulatory subunits on each end. Polyubiquitination of proteins triggers degradation by the 26S proteasome complex (*18*). The proteasome has become a popular target for drug therapy, since inhibiting the proteasome can be used for treatment of inflammatory diseases and cancer (*381*); on the other hand, activation of the proteasome has therapeutic use in neurodegenerative diseases (*382*).

Synchronized synthesis and degradation of regulatory proteins is necessary for cells to function and progress through the cell cycle. In the case of tumor development, if the degradation of these proteins can be blocked, apoptosis will ensue. Rapidly dividing cancer cells are more susceptible to proteasomal inhibition since they have more disruptions in their normal regulatory pathways than noncancerous cells (*383*). Bortezomib is the first small molecule inhibitor of the ubiquitin-protease system to be approved for treatment of multiple myeloma (*384, 385*). While bortezomib targets the 20S subunit of the proteasome, many other drugs are being developed to target other

components of the proteasome (*386, 387*) and other components (ubiquitin ligases, ubiquitin activating and conjugating enzymes) of the proteasomal degradation pathway (*388*). In addition, aberrant proteins could be sent to the proteasome for degradation. One focus of our laboratory is a gene therapy approach to capture oncogenic proteins and send them to the proteasome for degradation.

Future Perspectives

While unidirectional targeting of a therapeutic agent to single organelles has been well-studied and utilized, multi-directional drug targeting, utilizing multiple signals, represents a more sophisticated level of targeting which mimics the complexity of nature. In nature, many signal transducing proteins are not simply directed to one compartment of a cell. They may interact with other proteins in multiple compartments of a cell. For example, p14/19 ARF protein can bind Mdm2 (a nucleocytoplasmic shuttling protein) to inhibit degradation of p53 by Mdm2. The p14/19 ARF protein also contains a nucleolar signal, which can re-direct the p14/19 ARF-Mdm2 complex to the nucleolus (*389-391*).

While we have already demonstrated that unidirectional targeting of an oncogene can dramatically alter its function (*4*), our ultimate goal is to utilize our protein switch technology to capture an endogenous protein in one compartment, and move it to another compartment (*6, 7*). Our laboratory is pursuing this approach for treatment of chronic myelogenous leukemia (CML). Bcr-Abl, the causative agent of CML, is found in the cytoplasm of CML cells, where it sends oncogenic signals to other proteins in the cytoplasm. If endogenous Bcr-Abl can be captured and dragged to the nucleus of cells, apoptosis will ensue due to loss of oncogenic signaling in the cytoplasm, and gain of

apoptotic function in the nucleus. Like nature, the protein switch was designed to regulate cellular proteins by changing their location and hence their function. The protein switch has the advantage of being regulated by an externally added ligand. The protein switch contains a dimerization domain (to capture the endogenous protein of interest), a nuclear export signal (conferring cytoplasmic localization), and a ligand-regulated nuclear import signal. Upon addition of ligand, the protein switch is designed to translocate from the cytoplasm to the nucleus, and drag its dimerization partner with it.

Other groundbreaking studies have included the concept of delivering drugs to multiple compartments (across multiple barriers) by mimicking viral delivery. A review by Wagner (*392*) discusses mimicking viral delivery of DNA to the cytoplasm, and into the nucleus. There are even virus-mimetic nanogels that can deliver drugs to target and neighboring cells (*67*).

The future of drug delivery to organelles lies in our ability to more closely mimic nature, or even improve on nature. Spatial placement of signaling sequences in a protein, balancing the strengths of different signals against each other, and the masking/unmasking of signals due to conformational changes in an engineered protein or therapeutic have not been fully explored or understood. The ability to harness genetic codes, signal sequences, targeting motifs, or protein-protein interactions will lead to enormous complexity and diversity for drug delivery.

Executive Summary

1. For maximum therapeutic effect and minimal side effects, drugs need to be delivered to the appropriate location within the cell.

2. The cytoplasm and nucleus are the most widely targeted organelles, and the most well-studied.
3. Cytosolic targeting is crucial before most drugs can reach any organelle within the cell.
4. Drug carriers are still in progress to overcome barriers for cytosolic delivery and endosomal escape.
5. Localization and export of proteins to and from the nucleus can also be blocked using molecules that inhibit either the import or export mechanism.
6. Gene delivery mainly targets the nucleus, while a few studies are targeting DNA to the mitochondria.
7. Targeting other organelles is under investigation, especially the mitochondria due to its rule in oxidative stress and apoptosis.
8. Manipulating current therapeutics with specific targeting signals ensures accumulation in certain compartments within the cell.
9. Capturing and sending oncogenic proteins to the proteasome is a promising approach for cancer therapy.
10. Proteins synthesized at the ER are destined for intracellular organelles and cell membrane.

Newer technologies (protein switch, virus-like delivery systems) mimic nature and allow for a more sophisticated approach to drug delivery. Harnessing different methods of targeting multiple organelles in a cell will lead to better drug delivery and improvements in disease therapy.

Acknowledgments

This work was funded by NIH R01 CA129528 and DOD 55900381.

References

1. Heemels, M. T. (1999) Medicine Nobel goes to pioneer of protein guidance mechanisms, *Nature 401*, 625.

2. Hagmen, M. (1999) Protein ZIP codes make Nobel journey, *Science 286*, 666.

3. Shields, D. (2001) Gunter Blobel--still passionate after all these years, *Trends Cell Biol. 11*, 349-350.

4. Dixon, A. S., Kakar, M., Schneider, K. M., Constance, J. E., Paullin, B. C., and Lim, C. S. (2009) Controlling subcellular localization to alter function: Sending oncogenic Bcr-Abl to the nucleus causes apoptosis, *J. Control. Release 140*, 245-249.

5. Davis, J. R., Kakar, M., and Lim, C. S. (2007) Controlling protein compartmentalization to overcome disease, *Pharm. Res. 24*, 17-27.

6. Kakar, M., Davis, J. R., Kern, S. E., and Lim, C. S. (2007) Optimizing the protein switch: altering nuclear import and export signals, and ligand binding domain, *J. Control. Release 120*, 220-232.

7. Kanwal, C., Mu, S., Kern, S. E., and Lim, C. S. (2004) Bidirectional on/off switch for controlled targeting of proteins to subcellular compartments, *J. Control. Release 98*, 379-393.

8. Yaseen, M. A., Yu, J., Jung, B., Wong, M. S., and Anvari, B. (2009) Biodistribution of encapsulated indocyanine green in healthy mice, *Mol. Pharm. 6*, 1321-1332.

9. Li, S. D., and Huang, L. (2008) Pharmacokinetics and biodistribution of nanoparticles, *Mol. Pharm. 5*, 496-504.

10. Alexis, F., Pridgen, E., Molnar, L. K., and Farokhzad, O. C. (2008) Factors affecting the clearance and biodistribution of polymeric nanoparticles, *Mol. Pharm. 5*, 505-515.

11. Owens, D. E., 3rd, and Peppas, N. A. (2006) Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, *Int. J. Pharm. 307*, 93-102.

12. Connor, J., Norley, N., and Huang, L. (1986) Biodistribution of pH-sensitive immunoliposomes, *Biochim. Biophys. Acta. 884*, 474-481.

13. Liu, D., and Huang, L. (1989) Role of cholesterol in the stability of pH-sensitive, large unilamellar liposomes prepared by the detergent-dialysis method, *Biochim. Biophys. Acta. 981*, 254-260.

14. Liu, D., and Huang, L. (1990) pH-sensitive, plasma-stable liposomes with relatively prolonged residence in circulation, *Biochim. Biophys. Acta. 1022*, 348-354.

15. Collins, D., Litzinger, D. C., and Huang, L. (1990) Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols, *Biochim. Biophys. Acta. 1025*, 234-242.

16. Liu, D., Mori, A., and Huang, L. (1991) Large liposomes containing ganglioside GM1 accumulate effectively in spleen, *Biochim. Biophys. Acta. 1066*, 159-165.

17. Kono, K., Igawa, T., and Takagishi, T. (1997) Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative, *Biochim. Biophys. Acta. 1325*, 143-154.

18. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2008) *Mol. Biol. Cell*, 5th ed., Garland Science, New York.

19. Guillem, V. M., and Alino, S. F. (2004) Transfection pathways of nonspecific and targeted PEI-polyplexes, *Gene Ther. Mol. Biol. 8*, 369-384.

20. Poon, G. M., and Gariepy, J. (2007) Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells, *Biochem. Soc. Trans. 35*, 788-793.

21. Nitin, N., Santangelo, P. J., Kim, G., Nie, S., and Bao, G. (2004) Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells, *Nucleic Acids Res. 32*, e58.

22. Ziegler, A., Nervi, P., Durrenberger, M., and Seelig, J. (2005) The cationic cell-penetrating peptide CPP(TAT) derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence, *Biochem. 44*, 138-148.

23. Green, M., and Loewenstein, P. M. (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein, *Cell 55*, 1179-1188.

24. Frankel, A. D., and Pabo, C. O. (1988) Cellular uptake of the tat protein from human immunodeficiency virus, *Cell 55*, 1189-1193.

25. Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) Arginine-rich peptides. An abundant source of membrane-permeable

peptides having potential as carriers for intracellular protein delivery, *J. Biol. Chem. 276*, 5836-5840.

26. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse, *Science 285*, 1569-1572.

27. Heitz, F., Morris, M. C., and Divita, G. (2009) Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics, *Br. J. Pharmacol. 157*, 195-206.

28. Nori, A., Jensen, K. D., Tijerina, M., Kopeckova, P., and Kopecek, J. (2003) Tat-conjugated synthetic macromolecules facilitate cytoplasmic drug delivery to human ovarian carcinoma cells, *Bioconjug. Chem. 14*, 44-50.

29. Sethuraman, V. A., and Bae, Y. H. (2007) TAT peptide-based micelle system for potential active targeting of anti-cancer agents to acidic solid tumors, *J. Control. Release 118*, 216-224.

30. Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, *Nat. Med. 10*, 310-315.

31. Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, *J. Biol. Chem. 278*, 585-590.

32. Jarver, P., and Langel, U. (2004) The use of cell-penetrating peptides as a tool for gene regulation, *Drug Discov. Today 9*, 395-402.

33. Torchilin, V. P. (2006) Recent approaches to intracellular delivery of drugs and DNA and organelle targeting, *Annu. Rev. Biomed. Eng. 8*, 343-375.

34. Zhang, C., Tang, N., Liu, X., Liang, W., Xu, W., and Torchilin, V. P. (2006) siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene, *J. Control. Release 112*, 229-239.

35. Bengali, Z., Pannier, A. K., Segura, T., Anderson, B. C., Jang, J. H., Mustoe, T. A., and Shea, L. D. (2005) Gene delivery through cell culture substrate adsorbed DNA complexes, *Biotechnol. Bioeng. 90*, 290-302.

36. Panyam, J., and Labhasetwar, V. (2003) Biodegradable nanoparticles for drug and gene delivery to cells and tissue, *Adv. Drug Deliv. Rev. 55*, 329-347.

37. Vasir, J. K., and Labhasetwar, V. (2007) Biodegradable nanoparticles for cytosolic delivery of therapeutics, *Adv. Drug Deliv. Rev. 59*, 718-728.

38. Chu, C. J., Dijkstra, J., Lai, M. Z., Hong, K., and Szoka, F. C. (1990) Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture, *Pharm. Res. 7*, 824-834.

39. Schroit, A. J., Madsen, J., and Nayar, R. (1986) Liposome-cell interactions: in vitro discrimination of uptake mechanism and in vivo targeting strategies to mononuclear phagocytes, *Chem. Phys. Lipids 40*, 373-393.

40. Liu, D. X., and Huang, L. (1989) Small, but not large, unilamellar liposomes composed of dioleoylphosphatidylethanolamine and oleic acid can be stabilized by human plasma, *Biochem. 28*, 7700-7707.

41. Ellens, H., Bentz, J., and Szoka, F. C. (1984) pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact, *Biochem. 23*, 1532-1538.

42. Wei, H., Zhang, X. Z., Chen, W. Q., Cheng, S. X., and Zhuo, R. X. (2007) Self-assembled thermosensitive micelles based on poly(L-lactide-star block-N-isopropylacrylamide) for drug delivery, *J. Biomed. Mater. Res. A 83*, 980-989.

43. Toti, U. S., Moon, S. H., Kim, H. Y., Jun, Y. J., Kim, B. M., Park, Y. M., Jeong, B., and Sohn, Y. S. (2007) Thermosensitive and biocompatible cyclotriphosphazene micelles, *J. Control. Release 119*, 34-40.

44. Zhou, Y., Yan, D., Dong, W., and Tian, Y. (2007) Temperature-responsive phase transition of polymer vesicles: real-time morphology observation and molecular mechanism, *J. Phys. Chem. B 111*, 1262-1270.

45. Yang, M., Ding, Y., Zhang, L., Qian, X., Jiang, X., and Liu, B. (2007) Novel thermosensitive polymeric micelles for docetaxel delivery, *J. Biomed. Mater. Res. A 81*, 847-857.

46. Wei, H., Zhang, X., Cheng, C., Cheng, S. X., and Zhuo, R. X. (2007) Self-assembled, thermosensitive micelles of a star block copolymer based on PMMA and PNIPAAm for controlled drug delivery, *Biomaterials 28*, 99-107.

47. Rangelov, S., Dimitrov, P., and Tsvetanov, C. B. (2005) Mixed block copolymer aggregates with tunable temperature behavior, *J Phys Chem B 109*, 1162-1167.

48. Bae, K. H., Choi, S. H., Park, S. Y., Lee, Y., and Park, T. G. (2006) Thermosensitive pluronic micelles stabilized by shell cross-linking with gold nanoparticles, *Langmuir 22*, 6380-6384.

49. Soga, O., van Nostrum, C. F., Fens, M., Rijcken, C. J., Schiffelers, R. M., Storm, G., and Hennink, W. E. (2005) Thermosensitive and biodegradable polymeric micelles for paclitaxel delivery, *J. Control. Release 103*, 341-353.

50. Jeong, B., Bae, Y. H., and Kim, S. W. (2000) Drug release from biodegradable injectable thermosensitive hydrogel of PEG-PLGA-PEG triblock copolymers, *J. Control. Release 63*, 155-163.

51. Yockman, J. W., Kastenmeier, A., Erickson, H. M., Brumbach, J. G., Whitten, M. G., Albanil, A., Li, D. Y., Kim, S. W., and Bull, D. A. (2008) Novel polymer carriers and gene constructs for treatment of myocardial ischemia and infarction, *J. Control. Release 132*, 260-266.

52. Mossalam, M., Jeong, J. H., Abel, E. D., Kim, S. W., and Kim, Y. H. (2008) Reversal of oxidative stress in endothelial cells by controlled release of adiponectin, *J. Control. Release 130*, 234-237.

53. Li, Z., Zhang, Y., Wurtz, W., Lee, J. K., Malinin, V. S., Durwas-Krishnan, S., Meers, P., and Perkins, W. R. (2008) Characterization of nebulized liposomal amikacin (Arikace) as a function of droplet size, *J. Aerosol Med. Pulm. Drug Deliv 21*, 245-254.

54. Venkataram, S., Awni, W. M., Jordan, K., and Rahman, Y. E. (1990) Pharmacokinetics of two alternative dosage forms for cyclosporine: liposomes and intralipid, *J. Pharm. Sci. 79*, 216-219.

55. James, N. D., Coker, R. J., Tomlinson, D., Harris, J. R., Gompels, M., Pinching, A. J., and Stewart, J. S. (1994) Liposomal doxorubicin (Doxil): an effective new treatment for Kaposi's sarcoma in AIDS, *Clin. Oncol. (R Coll. Radiol.) 6*, 294-296.

56. Lutwyche, P., Cordeiro, C., Wiseman, D. J., St-Louis, M., Uh, M., Hope, M. J., Webb, M. S., and Finlay, B. B. (1998) Intracellular delivery and antibacterial activity of gentamicin encapsulated in pH-sensitive liposomes, *Antimicrob Agents Chemother 42*, 2511-2520.

57. Han, H. D., Shin, B. C., and Choi, H. S. (2006) Doxorubicin-encapsulated thermosensitive liposomes modified with poly(N-isopropylacrylamide-co-acrylamide): drug release behavior and stability in the presence of serum, *Eur. J. Pharm. Biopharm. 62*, 110-116.

58. Pradhan, P., Banerjee, R., Bahadur, D., Koch, C., Mykhaylyk, O., and Plank, C. (2010) Targeted magnetic liposomes loaded with Doxorubicin, *Methods Mol. Biol. 605*, 279-293.

59. Pradhan, P., Giri, J., Rieken, F., Koch, C., Mykhaylyk, O., Doblinger, M., Banerjee, R., Bahadur, D., and Plank, C. (2009) Targeted temperature sensitive magnetic liposomes for thermo-chemotherapy, *J. Control. Release 142*, 108-121.

60. Yokoyama, M., Miyauchi, M., Yamada, N., Okano, T., Sakurai, Y., Kataoka, K., and Inoue, S. (1990) Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer, *Cancer Res. 50*, 1693-1700.

61. Alani, A. W., Bae, Y., Rao, D. A., and Kwon, G. S. (2010) Polymeric micelles for the pH-dependent controlled, continuous low dose release of paclitaxel, *Biomaterials 31*, 1765-1772.

62. Bae, Y., Nishiyama, N., and Kataoka, K. (2007) In vivo antitumor activity of the folate-conjugated pH-sensitive polymeric micelle selectively releasing adriamycin in the intracellular acidic compartments, *Bioconjug. Chem. 18*, 1131-1139.

63. Masaki, T., Rathi, R., Zentner, G., Leypoldt, J. K., Mohammad, S. F., Burns, G. L., Li, L., Zhuplatov, S., Chirananthavat, T., Kim, S. J., Kern, S., Holman, J., Kim, S. W., and Cheung, A. K. (2004) Inhibition of neointimal hyperplasia in vascular grafts by sustained perivascular delivery of paclitaxel, *Kidney Int. 66*, 2061-2069.

64. Nori, A., Jensen, K. D., Tijerina, M., Kopeckova, P., and Kopecek, J. (2003) Subcellular trafficking of HPMA copolymer-Tat conjugates in human ovarian carcinoma cells, *J. Control. Release 91*, 53-59.

65. Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, *PNAS USA 92*, 7297-7301.

66. Jeong, J. H., Song, S. H., Lim, D. W., Lee, H., and Park, T. G. (2001) DNA transfection using linear poly(ethylenimine) prepared by controlled acid hydrolysis of poly(2-ethyl-2-oxazoline), *J. Control. Release 73*, 391-399.

67. Lee, E. S., Kim, D., Youn, Y. S., Oh, K. T., and Bae, Y. H. (2008) A virus-mimetic nanogel vehicle, *Angew. Chem. Int. Ed. Engl. 47*, 2418-2421.

68. Kakar, M., Cadwallader, A. B., Davis, J. R., and Lim, C. S. (2007) Signal sequences for targeting of gene therapy products to subcellular compartments: the role of CRM1 in nucleocytoplasmic shuttling of the protein switch, *Pharm. Res. 24*, 2146-2155.

69. Meunier, L., Mayer, R., Monsigny, M., and Roche, A. C. (1999) The nuclear export signal-dependent localization of oligonucleopeptides enhances the

inhibition of the protein expression from a gene transcribed in cytosol, *Nucleic Acids Res 27*, 2730-2736.

70. Ho, R. J., Rouse, B. T., and Huang, L. (1986) Target-sensitive immunoliposomes: preparation and characterization, *Biochem. 25*, 5500-5506.

71. Straubinger, R. M., Duzgunes, N., and Papahadjopoulos, D. (1985) pH-sensitive liposomes mediate cytoplasmic delivery of encapsulated macromolecules, *FEBS Lett. 179*, 148-154.

72. Leamon, C. P., and Low, P. S. (1991) Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis, *PNAS USA 88*, 5572-5576.

73. Thorstensen, K., and Romslo, I. (1993) The transferrin receptor: its diagnostic value and its potential as therapeutic target, *Scand. J. Clin. Lab Invest. Suppl. 215*, 113-120.

74. Chung, N. S., and Wasan, K. M. (2004) Potential role of the low-density lipoprotein receptor family as mediators of cellular drug uptake, *Adv. Drug Deliv. Rev. 56*, 1315-1334.

75. Vyas, S. P., Singh, A., and Sihorkar, V. (2001) Ligand-receptor-mediated drug delivery: an emerging paradigm in cellular drug targeting, *Crit. Rev. Ther. Drug Carrier Syst. 18*, 1-76.

76. Gorlich, D., and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm, *Annu. Rev. Cell Dev. Biol. 15*, 607-660.

77. Akinc, A., Thomas, M., Klibanov, A. M., and Langer, R. (2005) Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis, *J. Gene Med. 7*, 657-663.

78. Chollet, P., Favrot, M. C., Hurbin, A., and Coll, J. L. (2002) Side-effects of a systemic injection of linear polyethylenimine-DNA complexes, *J. Gene Med. 4*, 84-91.

79. Park, T. G., Jeong, J. H., and Kim, S. W. (2006) Current status of polymeric gene delivery systems, *Adv. Drug Deliv. Rev. 58*, 467-486.

80. Masotti, A., Moretti, F., Mancini, F., Russo, G., Di Lauro, N., Checchia, P., Marianecci, C., Carafa, M., Santucci, E., and Ortaggi, G. (2007) Physicochemical and biological study of selected hydrophobic polyethylenimine-based polycationic liposomes and their complexes with DNA, *Bioorg. Med. Chem. 15*, 1504-1515.

81. Neu, M., Fischer, D., and Kissel, T. (2005) Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives, *J. Gene Med. 7*, 992-1009.

82. Wang, Y., Chen, P., and Shen, J. (2006) The development and characterization of a glutathione-sensitive cross-linked polyethylenimine gene vector, *Biomaterials 27*, 5292-5298.

83. Zintchenko, A., Philipp, A., Dehshahri, A., and Wagner, E. (2008) Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity, *Bioconjug. Chem. 19*, 1448-1455.

84. Yu, J. H., Quan, J. S., Huang, J., Wang, C. Y., Sun, B., Nah, J. W., Cho, M. H., and Cho, C. S. (2009) Alpha,beta-poly(L-aspartate-graft-PEI): A pseudo-branched PEI as a gene carrier with low toxicity and high transfection efficiency, *Acta. Biomater. 5*, 2485-2494.

85. Duvall, C. L., Convertine, A., Benoit, D. S., Hoffman, A. S., and Stayton, P. (2009) Intracellular Delivery of a Proapoptotic Peptide via Conjugation to a RAFT Synthesized Endosomolytic Polymer, *Mol. Pharm*. *7*, 468-476.

86. Murthy, N., Campbell, J., Fausto, N., Hoffman, A. S., and Stayton, P. S. (2003) Bioinspired pH-responsive polymers for the intracellular delivery of biomolecular drugs, *Bioconjug. Chem. 14*, 412-419.

87. Bareford, L. M., and Swaan, P. W. (2007) Endocytic mechanisms for targeted drug delivery, *Adv. Drug Deliv. Rev. 59*, 748-758.

88. Gary-Bobo, M., Nirde, P., Jeanjean, A., Morere, A., and Garcia, M. (2007) Mannose 6-phosphate receptor targeting and its applications in human diseases, *Curr. Med. Chem. 14*, 2945-2953.

89. LeBowitz, J. H., Grubb, J. H., Maga, J. A., Schmiel, D. H., Vogler, C., and Sly, W. S. (2004) Glycosylation-independent targeting enhances enzyme delivery to lysosomes and decreases storage in mucopolysaccharidosis type VII mice, *PNAS USA 101*, 3083-3088.

90. Feldherr, C. M. (1962) The nuclear annuli as pathways for nucleocytoplasmic exchanges, *J, Cell Biol. 14*, 65-72.

91. Mans, B. J., Anantharaman, V., Aravind, L., and Koonin, E. V. (2004) Comparative genomics, evolution and origins of the nuclear envelope and nuclear pore complex, *Cell Cycle 3*, 1612-1637.

92. Jekely, G. (2005) Glimpsing over the event horizon: evolution of nuclear pores and envelope, *Cell Cycle 4*, 297-299.

93. Feldherr, C., Akin, D., Littlewood, T., and Stewart, M. (2002) The molecular mechanism of translocation through the nuclear pore complex is highly conserved, *J. Cell Sci. 115*, 2997-3005.

94. Harel, A., and Forbes, D. J. (2004) Importin beta: conducting a much larger cellular symphony, *Mol. Cell 16*, 319-330.

95. Wente, S. R. (2000) Gatekeepers of the nucleus, *Science 288*, 1374-1377.

96. Ribbeck, K., and Gorlich, D. (2001) Kinetic analysis of translocation through nuclear pore complexes, *EMBO J. 20*, 1320-1330.

97. Akey, C. W., and Radermacher, M. (1993) Architecture of the Xenopus nuclear pore complex revealed by three-dimensional cryo-electron microscopy, *J. Cell Biol. 122*, 1-19.

98. Allen, T. D., Cronshaw, J. M., Bagley, S., Kiseleva, E., and Goldberg, M. W. (2000) The nuclear pore complex: mediator of translocation between nucleus and cytoplasm, *J. Cell Sci. 113 (Pt 10)*, 1651-1659.

99. Stoffler, D., Fahrenkrog, B., and Aebi, U. (1999) The nuclear pore complex: from molecular architecture to functional dynamics, *Curr. Opin. Cell Biol. 11*, 391-401.

100. Seedorf, M., Damelin, M., Kahana, J., Taura, T., and Silver, P. A. (1999) Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase, *Mol. Cell Biol. 19*, 1547-1557.

101. Shah, S., Tugendreich, S., and Forbes, D. (1998) Major binding sites for the nuclear import receptor are the internal nucleoporin Nup153 and the adjacent nuclear filament protein Tpr, *J. Cell Biol. 141*, 31-49.

102. Iovine, M. K., and Wente, S. R. (1997) A nuclear export signal in Kap95p is required for both recycling the import factor and interaction with the nucleoporin GLFG repeat regions of Nup116p and Nup100p, *J. Cell Biol. 137*, 797-811.

103. Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995) The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor, *J. Cell Biol. 131*, 1699-1713.

104. Lyman, S. K., Guan, T., Bednenko, J., Wodrich, H., and Gerace, L. (2002) Influence of cargo size on Ran and energy requirements for nuclear protein import, *J. Cell Biol. 159*, 55-67.

105. Turpin, P., Ossareh-Nazari, B., and Dargemont, C. (1999) Nuclear transport and transcriptional regulation, *FEBS Lett. 452*, 82-86.

106. Paine, P. L., Moore, L. C., and Horowitz, S. B. (1975) Nuclear envelope permeability, *Nature 254*, 109-114.

107. Talcott, B., and Moore, M. S. (1999) Getting across the nuclear pore complex, *Trends Cell Biol. 9*, 312-318.

108. Poon, I. K., and Jans, D. A. (2005) Regulation of nuclear transport: central role in development and transformation?, *Traffic 6*, 173-186.

109. Feldherr, C. M., and Akin, D. (1997) The location of the transport gate in the nuclear pore complex, *J. Cell Sci. 110 (Pt 24)*, 3065-3070.

110. Weis, K. (2003) Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle, *Cell 112*, 441-451.

111. Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) Sequence requirements for nuclear location of simian virus 40 large-T antigen, *Nature 311*, 33-38.

112. Hodel, M. R., Corbett, A. H., and Hodel, A. E. (2001) Dissection of a nuclear localization signal, *J. Biol. Chem. 276*, 1317-1325.

113. Efthymiadis, A., Shao, H., Hubner, S., and Jans, D. A. (1997) Kinetic characterization of the human retinoblastoma protein bipartite nuclear localization sequence (NLS) in vivo and in vitro. A comparison with the SV40 large T-antigen NLS, *J. Biol. Chem. 272*, 22134-22139.

114. Gorlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import, *Cell 79*, 767-778.

115. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha, *Cell 94*, 193-204.

116. Chook, Y. M., and Blobel, G. (2001) Karyopherins and nuclear import, *Curr. Opin. Struct. Biol. 11*, 703-715.

117. Gorlich, D., Henklein, P., Laskey, R. A., and Hartmann, E. (1996) A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus, *EMBO J. 15*, 1810-1817.

118. Bayliss, R., Littlewood, T., and Stewart, M. (2000) Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking, *Cell 102*, 99-108.

119. Azuma, Y., and Dasso, M. (2000) The role of Ran in nuclear function, *Curr. Opin. Cell Biol. 12*, 302-307.

120. Vetter, I. R., Nowak, C., Nishimoto, T., Kuhlmann, J., and Wittinghofer, A. (1999) Structure of a Ran-binding domain complexed with Ran bound to a GTP analogue: implications for nuclear transport, *Nature 398*, 39-46.

121. Kohler, M., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., Haller, H., Gorlich, D., and Hartmann, E. (1999) Evidence for distinct substrate specificities of importin alpha family members in nuclear protein import, *Mol. Cell Biol. 19*, 7782-7791.

122. Lukacs, G. L., Haggie, P., Seksek, O., Lechardeur, D., Freedman, N., and Verkman, A. S. (2000) Size-dependent DNA mobility in cytoplasm and nucleus, *J. Biol. Chem. 275*, 1625-1629.

123. Dauty, E., and Verkman, A. S. (2005) Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm: a new barrier for non-viral gene delivery, *J. Biol. Chem. 280*, 7823-7828.

124. Dohner, K., Nagel, C. H., and Sodeik, B. (2005) Viral stop-and-go along microtubules: taking a ride with dynein and kinesins, *Trends Microbiol. 13*, 320-327.

125. Greber, U. F., and Way, M. (2006) A superhighway to virus infection, *Cell 124*, 741-754.

126. Welte, M. A. (2004) Bidirectional transport along microtubules, *Curr. Biol. 14*, R525-537.

127. Mastrobattista, E., van der Aa, M. A., Hennink, W. E., and Crommelin, D. J. (2006) Artificial viruses: a nanotechnological approach to gene delivery, *Nat. Rev. Drug Discov 5*, 115-121.

128. van der Aa, M. A., Mastrobattista, E., Oosting, R. S., Hennink, W. E., Koning, G. A., and Crommelin, D. J. (2006) The nuclear pore complex: the gateway to successful nonviral gene delivery, *Pharm. Res. 23*, 447-459.

129. Lim, R. Y., and Fahrenkrog, B. (2006) The nuclear pore complex up close, *Curr. Opin. Cell Biol. 18*, 342-347.

130. Pante, N., and Kann, M. (2002) Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm, *Mol. Biol. Cell 13*, 425-434.

131. Chan, C. K., Senden, T., and Jans, D. A. (2000) Supramolecular structure and nuclear targeting efficiency determine the enhancement of transfection by modified polylysines, *Gene Ther. 7*, 1690-1697.

132. Chan, C. K., and Jans, D. A. (2002) Using nuclear targeting signals to enhance non-viral gene transfer, *Immunol. Cell Biol. 80*, 119-130.

133. Pouton, C. W., Wagstaff, K. M., Roth, D. M., Moseley, G. W., and Jans, D. A. (2007) Targeted delivery to the nucleus, *Adv. Drug Deliv. Rev. 59*, 698-717.

134. Collas, P., and Alestrom, P. (1996) Nuclear localization signal of SV40 T antigen directs import of plasmid DNA into sea urchin male pronuclei in vitro, *Mol. Reprod. Dev. 45*, 431-438.

135. Collas, P., Husebye, H., and Alestrom, P. (1996) The nuclear localization sequence of the SV40 T antigen promotes transgene uptake and expression in zebrafish embryo nuclei, *Transgenic Res. 5*, 451-458.

136. Ritter, W., Plank, C., Lausier, J., Rudolph, C., Zink, D., Reinhardt, D., and Rosenecker, J. (2003) A novel transfecting peptide comprising a tetrameric nuclear localization sequence, *J. Mol. Med. 81*, 708-717.

137. Aronsohn, A. I., and Hughes, J. A. (1998) Nuclear localization signal peptides enhance cationic liposome-mediated gene therapy, *J. Drug Target 5*, 163-169.

138. Keller, M., Harbottle, R. P., Perouzel, E., Colin, M., Shah, I., Rahim, A., Vaysse, L., Bergau, A., Moritz, S., Brahimi-Horn, C., Coutelle, C., and Miller, A. D. (2003) Nuclear localisation sequence templated nonviral gene delivery vectors: investigation of intracellular trafficking events of LMD and LD vector systems, *Chembiochem. 4*, 286-298.

139. Chan, C. K., and Jans, D. A. (1999) Enhancement of polylysine-mediated transferrinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence, *Hum. Gene Ther. 10*, 1695-1702.

140. Subramanian, A., Ranganathan, P., and Diamond, S. L. (1999) Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells, *Nat. Biotechnol. 17*, 873-877.

141. Branden, L. J., Mohamed, A. J., and Smith, C. I. (1999) A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA, *Nat. Biotechnol. 17*, 784-787.

142. Cutrona, G., Boffa, L. C., Mariani, M. R., Matis, S., Damonte, G., Millo, E., Roncella, S., and Ferrarini, M. (2007) The peptide nucleic acid targeted to a regulatory sequence of the translocated c-myc oncogene in Burkitt's lymphoma

lacks immunogenicity: follow-up characterization of PNAEmu-NLS, *Oligonucleotides 17*, 146-150.

143. Rasmussen, F. W., Bendifallah, N., Zachar, V., Shiraishi, T., Fink, T., Ebbesen, P., Nielsen, P. E., and Koppelhus, U. (2006) Evaluation of transfection protocols for unmodified and modified peptide nucleic acid (PNA) oligomers, *Oligonucleotides 16*, 43-57.

144. Cogoi, S., Codognotto, A., Rapozzi, V., and Xodo, L. E. (2005) Antigene property of PNA conjugated to the nuclear localization signal peptide, *Nucleosides Nucleotides Nucleic Acids 24*, 971-974.

145. Cogoi, S., Codognotto, A., Rapozzi, V., Meeuwenoord, N., van der Marel, G., and Xodo, L. E. (2005) Transcription inhibition of oncogenic KRAS by a mutation-selective peptide nucleic acid conjugated to the PKKKRKV nuclear localization signal peptide, *Biochem. 44*, 10510-10519.

146. Roulon, T., Helene, C., and Escude, C. (2002) Coupling of a targeting peptide to plasmid DNA using a new type of padlock oligonucleotide, *Bioconjug. Chem. 13*, 1134-1139.

147. Ludtke, J. J., Zhang, G., Sebestyen, M. G., and Wolff, J. A. (1999) A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA, *J. Cell Sci. 112 (Pt 12)*, 2033-2041.

148. Sebestyen, M. G., Ludtke, J. J., Bassik, M. C., Zhang, G., Budker, V., Lukhtanov, E. A., Hagstrom, J. E., and Wolff, J. A. (1998) DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA, *Nat. Biotechnol. 16*, 80-85.

149. Ciolina, C., Byk, G., Blanche, F., Thuillier, V., Scherman, D., and Wils, P. (1999) Coupling of nuclear localization signals to plasmid DNA and specific interaction of the conjugates with importin alpha, *Bioconjug. Chem. 10*, 49-55.

150. Nagasaki, T., Myohoji, T., Tachibana, T., Futaki, S., and Tamagaki, S. (2003) Can nuclear localization signals enhance nuclear localization of plasmid DNA?, *Bioconjug. Chem. 14*, 282-286.

151. Neves, C., Byk, G., Scherman, D., and Wils, P. (1999) Coupling of a targeting peptide to plasmid DNA by covalent triple helix formation, *FEBS Lett. 453*, 41-45.

152. Zanta, M. A., Belguise-Valladier, P., and Behr, J. P. (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus, *PNAS USA 96*, 91-96.

153. Tanimoto, M., Kamiya, H., Minakawa, N., Matsuda, A., and Harashima, H. (2003) No enhancement of nuclear entry by direct conjugation of a nuclear localization signal peptide to linearized DNA, *Bioconjug. Chem. 14*, 1197-1202.

154. van der Aa, M. A., Koning, G. A., d'Oliveira, C., Oosting, R. S., Wilschut, K. J., Hennink, W. E., and Crommelin, D. J. (2005) An NLS peptide covalently linked to linear DNA does not enhance transfection efficiency of cationic polymer based gene delivery systems, *J. Gene Med. 7*, 208-217.

155. Johansson, P., Lindgren, T., Lundstrom, M., Holmstrom, A., Elgh, F., and Bucht, G. (2002) PCR-generated linear DNA fragments utilized as a hantavirus DNA vaccine, *Vaccine 20*, 3379-3388.

156. Carriere, M., Escriou, V., Savarin, A., and Scherman, D. (2003) Coupling of importin beta binding peptide on plasmid DNA: transfection efficiency is increased by modification of lipoplex's physico-chemical properties, *BMC Biotechnol. 3*, 14.

157. Chahine, M. N., and Pierce, G. N. (2009) Therapeutic targeting of nuclear protein import in pathological cell conditions, *Pharmacol. Rev. 61*, 358-372.

158. Snow, C. M., Senior, A., and Gerace, L. (1987) Monoclonal antibodies identify a group of nuclear pore complex glycoproteins, *J. Cell Biol. 104*, 1143-1156.

159. Finlay, D. R., Newmeyer, D. D., Price, T. M., and Forbes, D. J. (1987) Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores, *J. Cell Biol. 104*, 189-200.

160. Adam, E. J., and Adam, S. A. (1994) Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope, *J. Cell Biol. 125*, 547-555.

161. Kosugi, S., Hasebe, M., Entani, T., Takayama, S., Tomita, M., and Yanagawa, H. (2008) Design of peptide inhibitors for the importin alpha/beta nuclear import pathway by activity-based profiling, *Chem. Biol. 15*, 940-949.

162. Wolff, B., Sanglier, J. J., and Wang, Y. (1997) Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA, *Chem. Biol. 4*, 139-147.

163. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export, *Science 278*, 141-144.

164. Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998) Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1, *Exp. Cell Res. 242*, 540-547.

165. Mutka, S. C., Yang, W. Q., Dong, S. D., Ward, S. L., Craig, D. A., Timmermans, P. B., and Murli, S. (2009) Identification of nuclear export inhibitors with potent anticancer activity in vivo, *Cancer Res. 69*, 510-517.

166. Pieper, G. M., and Riaz ul, H. (1997) Activation of nuclear factor-kappaB in cultured endothelial cells by increased glucose concentration: prevention by calphostin C, *J. Cardiovasc. Pharmacol. 30*, 528-532.

167. Shoji, S., Furuishi, K., Ogata, A., Yamataka, K., Tachibana, K., Mukai, R., Uda, A., Harano, K., Matsushita, S., and Misumi, S. (1998) An allosteric drug, o,o'-bismyristoyl thiamine disulfide, suppresses HIV-1 replication through prevention of nuclear translocation of both HIV-1 Tat and NF-kappa B, *Biochem. Biophys. Res. Commun. 249*, 745-753.

168. Wong, E. T., and Tergaonkar, V. (2009) Roles of NF-kappaB in health and disease: mechanisms and therapeutic potential, *Clin. Sci. (Lond.) 116*, 451-465.

169. Hallhuber, M., Burkard, N., Wu, R., Buch, M. H., Engelhardt, S., Hein, L., Neyses, L., Schuh, K., and Ritter, O. (2006) Inhibition of nuclear import of calcineurin prevents myocardial hypertrophy, *Circ. Res. 99*, 626-635.

170. Colas, P., Cohen, B., Ko Ferrigno, P., Silver, P. A., and Brent, R. (2000) Targeted modification and transportation of cellular proteins, *PNAS USA 97*, 13720-13725.

171. Emmott, E., and Hiscox, J. A. (2009) Nucleolar targeting: the hub of the matter, *EMBO Rep. 10*, 231-238.

172. Birbach, A., Bailey, S. T., Ghosh, S., and Schmid, J. A. (2004) Cytosolic, nuclear and nucleolar localization signals determine subcellular distribution and activity of the NF-kappaB inducing kinase NIK, *J. Cell Sci. 117*, 3615-3624.

173. Boyne, J. R., and Whitehouse, A. (2006) Nucleolar trafficking is essential for nuclear export of intronless herpesvirus mRNA, *PNAS USA 103*, 15190-15195.

174. Weber, J. D., Kuo, M. L., Bothner, B., DiGiammarino, E. L., Kriwacki, R. W., Roussel, M. F., and Sherr, C. J. (2000) Cooperative signals governing ARF-mdm2 interaction and nucleolar localization of the complex, *Mol. Cell Biol. 20*, 2517-2528.

175. Horke, S., Reumann, K., Schweizer, M., Will, H., and Heise, T. (2004) Nuclear trafficking of La protein depends on a newly identified nucleolar localization signal and the ability to bind RNA, *J. Biol. Chem. 279*, 26563-26570.

176. Reed, M. L., Dove, B. K., Jackson, R. M., Collins, R., Brooks, G., and Hiscox, J. A. (2006) Delineation and modelling of a nucleolar retention signal in the coronavirus nucleocapsid protein, *Traffic 7*, 833-848.

177. Ginisty, H., Sicard, H., Roger, B., and Bouvet, P. (1999) Structure and functions of nucleolin, *J. Cell Sci. 112 (Pt 6)*, 761-772.

178. Korgaonkar, C., Hagen, J., Tompkins, V., Frazier, A. A., Allamargot, C., Quelle, F. W., and Quelle, D. E. (2005) Nucleophosmin (B23) targets ARF to nucleoli and inhibits its function, *Mol. Cell Biol. 25*, 1258-1271.

179. Gunawardena, S. R., Ruis, B. L., Meyer, J. A., Kapoor, M., and Conklin, K. F. (2008) NOM1 targets protein phosphatase I to the nucleolus, *J. Biol. Chem. 283*, 398-404.

180. Junera, H. R., Masson, C., Geraud, G., and Hernandez-Verdun, D. (1995) The three-dimensional organization of ribosomal genes and the architecture of the nucleoli vary with G1, S and G2 phases, *J. Cell Sci. 108 (Pt 11)*, 3427-3441.

181. Puvion-Dutilleul, F., Puvion, E., and Bachellerie, J. P. (1997) Early stages of pre-rRNA formation within the nucleolar ultrastructure of mouse cells studied by in situ hybridization with a 5'ETS leader probe, *Chromosoma 105*, 496-505.

182. Sirri, V., Hernandez-Verdun, D., and Roussel, P. (2002) Cyclin-dependent kinases govern formation and maintenance of the nucleolus, *J. Cell Biol. 156*, 969-981.

183. Rubbi, C. P., and Milner, J. (2003) Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses, *EMBO J. 22*, 6068-6077.

184. David-Pfeuty, T., Nouvian-Dooghe, Y., Sirri, V., Roussel, P., and Hernandez-Verdun, D. (2001) Common and reversible regulation of wild-type p53 function and of ribosomal biogenesis by protein kinases in human cells, *Oncogene 20*, 5951-5963.

185. Louvet, E., Junera, H. R., Le Panse, S., and Hernandez-Verdun, D. (2005) Dynamics and compartmentation of the nucleolar processing machinery, *Exp. Cell Res. 304*, 457-470.

186. Kalinichenko, V. V., Major, M. L., Wang, X., Petrovic, V., Kuechle, J., Yoder, H. M., Dennewitz, M. B., Shin, B., Datta, A., Raychaudhuri, P., and Costa, R. H. (2004) Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor, *Genes Dev. 18*, 830-850.

187. Wang, X., Kiyokawa, H., Dennewitz, M. B., and Costa, R. H. (2002) The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration, *PNAS USA 99*, 16881-16886.

188. McFarland, R., Taylor, R. W., and Turnbull, D. M. (2007) Mitochondrial disease--its impact, etiology, and pathology, *Curr. Top Dev. Biol. 77*, 113-155.

189. Taylor, R. W., and Turnbull, D. M. (2005) Mitochondrial DNA mutations in human disease, *Nat. Rev. Genet. 6*, 389-402.

190. Newmeyer, D. D., and Ferguson-Miller, S. (2003) Mitochondria: releasing power for life and unleashing the machineries of death, *Cell 112*, 481-490.

191. Trushina, E., and McMurray, C. T. (2007) Oxidative stress and mitochondrial dysfunction in neurodegenerative diseases, *Neuroscience 145*, 1233-1248.

192. Orrenius, S., Gogvadze, V., and Zhivotovsky, B. (2007) Mitochondrial oxidative stress: implications for cell death, *Annu. Rev. Pharmacol. Toxicol. 47*, 143-183.

193. Droge, W. (2002) Free radicals in the physiological control of cell function, *Physiol. Rev. 82*, 47-95.

194. Raha, S., and Robinson, B. H. (2000) Mitochondria, oxygen free radicals, disease and ageing, *Trends Biochem. Sci. 25*, 502-508.

195. Beckman, K. B., and Ames, B. N. (1998) The free radical theory of aging matures, *Physiol. Rev. 78*, 547-581.

196. Wallace, D. C. (1999) Mitochondrial diseases in man and mouse, *Science 283*, 1482-1488.

197. Finkel, T. (1998) Oxygen radicals and signaling, *Curr. Opin. Cell Biol. 10*, 248-253.

198. Finkel, T., and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of ageing, *Nature 408*, 239-247.

199. Shigenaga, M. K., Hagen, T. M., and Ames, B. N. (1994) Oxidative damage and mitochondrial decay in aging, *PNAS USA 91*, 10771-10778.

200. Ross, M. F., Kelso, G. F., Blaikie, F. H., James, A. M., Cocheme, H. M., Filipovska, A., Da Ros, T., Hurd, T. R., Smith, R. A., and Murphy, M. P. (2005) Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology, *Biochem. (Mosc.) 70*, 222-230.

201. Green, D. R., and Reed, J. C. (1998) Mitochondria and apoptosis, *Science 281*, 1309-1312.

202. Reed, J. C. (1997) Cytochrome c: can't live with it--can't live without it, *Cell 91*, 559-562.

203. Green, D. R., and Evan, G. I. (2002) A matter of life and death, *Cancer Cell 1*, 19-30.

204. Galluzzi, L., Morselli, E., Kepp, O., Tajeddine, N., and Kroemer, G. (2008) Targeting p53 to mitochondria for cancer therapy, *Cell Cycle 7*, 1949-1955.

205. Matsuyama, S., and Reed, J. C. (2000) Mitochondria-dependent apoptosis and cellular pH regulation, *Cell Death Differ. 7*, 1155-1165.

206. Galluzzi, L., Larochette, N., Zamzami, N., and Kroemer, G. (2006) Mitochondria as therapeutic targets for cancer chemotherapy, *Oncogene 25*, 4812-4830.

207. Antignani, A., and Youle, R. J. (2006) How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane?, *Curr. Opin. Cell Biol. 18*, 685-689.

208. Green, D. R., and Kroemer, G. (2004) The pathophysiology of mitochondrial cell death, *Science 305*, 626-629.

209. Ruffolo, S. C., and Shore, G. C. (2003) BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization, *J. Biol. Chem. 278*, 25039-25045.

210. Mukhopadhyay, A., and Weiner, H. (2007) Delivery of drugs and macromolecules to mitochondria, *Adv. Drug Deliv. Rev. 59*, 729-738.

211. Adlam, V. J., Harrison, J. C., Porteous, C. M., James, A. M., Smith, R. A., Murphy, M. P., and Sammut, I. A. (2005) Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury, *FASEB J. 19*, 1088-1095.

212. James, A. M., Sharpley, M. S., Manas, A. R., Frerman, F. E., Hirst, J., Smith, R. A., and Murphy, M. P. (2007) Interaction of the mitochondria-targeted antioxidant MitoQ with phospholipid bilayers and ubiquinone oxidoreductases, *J. Biol. Chem. 282*, 14708-14718.

213. Filipovska, A., Kelso, G. F., Brown, S. E., Beer, S. M., Smith, R. A., and Murphy, M. P. (2005) Synthesis and characterization of a triphenylphosphonium-

conjugated peroxidase mimetic. Insights into the interaction of ebselen with mitochondria, *J. Biol. Chem. 280*, 24113-24126.

214. Smith, R. A., Kelso, G. F., James, A. M., and Murphy, M. P. (2004) Targeting coenzyme Q derivatives to mitochondria, *Methods Enzymol. 382*, 45-67.

215. Kelso, G. F., Porteous, C. M., Coulter, C. V., Hughes, G., Porteous, W. K., Ledgerwood, E. C., Smith, R. A., and Murphy, M. P. (2001) Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties, *J. Biol. Chem. 276*, 4588-4596.

216. Asin-Cayuela, J., Manas, A. R., James, A. M., Smith, R. A., and Murphy, M. P. (2004) Fine-tuning the hydrophobicity of a mitochondria-targeted antioxidant, *FEBS Lett. 571*, 9-16.

217. Smith, R. A., Porteous, C. M., Coulter, C. V., and Murphy, M. P. (1999) Selective targeting of an antioxidant to mitochondria, *Eur. J. Biochem. 263*, 709-716.

218. D'Souza, G. G., Boddapati, S. V., and Weissig, V. (2005) Mitochondrial leader sequence--plasmid DNA conjugates delivered into mammalian cells by DQAsomes co-localize with mitochondria, *Mitochondrion 5*, 352-358.

219. D'Souza, G. G., Rammohan, R., Cheng, S. M., Torchilin, V. P., and Weissig, V. (2003) DQAsome-mediated delivery of plasmid DNA toward mitochondria in living cells, *J. Control. Release 92*, 189-197.

220. Endo, T., and Kohda, D. (2002) Functions of outer membrane receptors in mitochondrial protein import, *Biochim. Biophys. Acta. 1592*, 3-14.

221. Dolezal, P., Likic, V., Tachezy, J., and Lithgow, T. (2006) Evolution of the molecular machines for protein import into mitochondria, *Science 313*, 314-318.

222. Rehling, P., Brandner, K., and Pfanner, N. (2004) Mitochondrial import and the twin-pore translocase, *Nat. Rev. Mol. Cell Biol. 5*, 519-530.

223. Meisinger, C., Ryan, M. T., Hill, K., Model, K., Lim, J. H., Sickmann, A., Muller, H., Meyer, H. E., Wagner, R., and Pfanner, N. (2001) Protein import channel of the outer mitochondrial membrane: a highly stable Tom40-Tom22 core structure differentially interacts with preproteins, small tom proteins, and import receptors, *Mol. Cell Biol. 21*, 2337-2348.

224. Herrmann, J. M., and Neupert, W. (2000) Protein transport into mitochondria, *Curr. Opin. Microbiol. 3*, 210-214.

225. Koehler, C. M. (2004) New developments in mitochondrial assembly, *Annu. Rev. Cell Dev. Biol. 20*, 309-335.

226. Jiang, J., Stoyanovsky, D. A., Belikova, N. A., Tyurina, Y. Y., Zhao, Q., Tungekar, M. A., Kapralova, V., Huang, Z., Mintz, A. H., Greenberger, J. S., and Kagan, V. E. (2009) A mitochondria-targeted triphenylphosphonium-conjugated nitroxide functions as a radioprotector/mitigator, *Radiat. Res. 172*, 706-717.

227. Ngen, E. J., Rajaputra, P., and You, Y. (2009) Evaluation of delocalized lipophilic cationic dyes as delivery vehicles for photosensitizers to mitochondria, *Bioorg. Med. Chem. 17*, 6631-6640.

228. Boddapati, S. V., D'Souza, G. G., Erdogan, S., Torchilin, V. P., and Weissig, V. (2008) Organelle-targeted nanocarriers: specific delivery of liposomal ceramide to mitochondria enhances its cytotoxicity in vitro and in vivo, *Nano. Lett. 8*, 2559-2563.

229. Takaya, K., Higuchi, Y., Kitamoto, K., and Arioka, M. (2009) A cytosolic phospholipase A2-like protein in the filamentous fungus Aspergillus oryzae localizes to the intramembrane space of the mitochondria, *FEMS Microbiol. Lett. 301*, 201-209.

230. Shokolenko, I. N., Alexeyev, M. F., LeDoux, S. P., and Wilson, G. L. (2005) TAT-mediated protein transduction and targeted delivery of fusion proteins into mitochondria of breast cancer cells, *DNA Repair (Amst.) 4*, 511-518.

231. Meton, I., Egea, M., Fernandez, F., Eraso, M. C., and Baanante, I. V. (2004) The N-terminal sequence directs import of mitochondrial alanine aminotransferase into mitochondria, *FEBS Lett. 566*, 251-254.

232. Marchenko, N. D., Zaika, A., and Moll, U. M. (2000) Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling, *J. Biol. Chem. 275*, 16202-16212.

233. Horwich, A. L., Kalousek, F., Mellman, I., and Rosenberg, L. E. (1985) A leader peptide is sufficient to direct mitochondrial import of a chimeric protein, *EMBO J. 4*, 1129-1135.

234. Von Heijne, G. (1986) Mitochondrial targeting sequences may form amphiphilic helices, *EMBO J. 5*, 1335-1342.

235. Roise, D., and Schatz, G. (1988) Mitochondrial presequences, *J. Biol. Chem. 263*, 4509-4511.

236. Neupert, W., and Herrmann, J. M. (2007) Translocation of proteins into mitochondria, *Annu. Rev. Biochem. 76*, 723-749.

237. Mokranjac, D., and Neupert, W. (2005) Protein import into mitochondria, *Biochem. Soc. Trans. 33*, 1019-1023.

238. Wiedemann, N., Frazier, A. E., and Pfanner, N. (2004) The protein import machinery of mitochondria, *J. Biol. Chem. 279*, 14473-14476.

239. Bohni, P. C., Daum, G., and Schatz, G. (1983) Import of proteins into mitochondria. Partial purification of a matrix-located protease involved in cleavage of mitochondrial precursor polypeptides, *J. Biol. Chem. 258*, 4937-4943.

240. Schmidt, B., Wachter, E., Sebald, W., and Neupert, W. (1984) Processing peptidase of Neurospora mitochondria. Two-step cleavage of imported ATPase subunit 9, *Eur. J. Biochem. 144*, 581-588.

241. Folsch, H., Guiard, B., Neupert, W., and Stuart, R. A. (1996) Internal targeting signal of the BCS1 protein: a novel mechanism of import into mitochondria, *EMBO J. 15*, 479-487.

242. Sirrenberg, C., Endres, M., Folsch, H., Stuart, R. A., Neupert, W., and Brunner, M. (1998) Carrier protein import into mitochondria mediated by the intermembrane proteins Tim10/Mrs11 and Tim12/Mrs5, *Nature 391*, 912-915.

243. Rodriguez-Cousino, N., Nargang, F. E., Baardman, R., Neupert, W., Lill, R., and Court, D. A. (1998) An import signal in the cytosolic domain of the Neurospora mitochondrial outer membrane protein TOM22, *J. Biol. Chem. 273*, 11527-11532.

244. Keil, P., and Pfanner, N. (1993) Insertion of MOM22 into the mitochondrial outer membrane strictly depends on surface receptors, *FEBS Lett. 321*, 197-200.

245. Mayer, A., Lill, R., and Neupert, W. (1993) Translocation and insertion of precursor proteins into isolated outer membranes of mitochondria, *J. Cell Biol. 121*, 1233-1243.

246. Pfanner, N., Hoeben, P., Tropschug, M., and Neupert, W. (1987) The carboxyl-terminal two-thirds of the ADP/ATP carrier polypeptide contains sufficient information to direct translocation into mitochondria, *J. Biol. Chem. 262*, 14851-14854.

247. Vestweber, D., and Schatz, G. (1989) DNA-protein conjugates can enter mitochondria via the protein import pathway, *Nature 338*, 170-172.

248. Seibel, M., Bachmann, C., Schmiedel, J., Wilken, N., Wilde, F., Reichmann, H., Isaya, G., Seibel, P., and Pfeiler, D. (1999) Processing of artificial peptide-DNA-conjugates by the mitochondrial intermediate peptidase (MIP), *Biol. Chem. 380*, 961-967.

249. Mukhopadhyay, A., Ni, L., Yang, C. S., and Weiner, H. (2005) Bacterial signal peptide recognizes HeLa cell mitochondrial import receptors and functions as a mitochondrial leader sequence, *Cell Mol. Life Sci. 62*, 1890-1899.

250. Srivastava, S., and Moraes, C. T. (2001) Manipulating mitochondrial DNA heteroplasmy by a mitochondrially targeted restriction endonuclease, *Hum. Mol. Genet. 10*, 3093-3099.

251. Tomita, Y., Marchenko, N., Erster, S., Nemajerova, A., Dehner, A., Klein, C., Pan, H., Kessler, H., Pancoska, P., and Moll, U. M. (2006) WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization, *J. Biol. Chem. 281*, 8600-8606.

252. Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) p53 has a direct apoptogenic role at the mitochondria, *Mol. Cell. 11*, 577-590.

253. Erster, S., Mihara, M., Kim, R. H., Petrenko, O., and Moll, U. M. (2004) In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation, *Mol. Cell. Biol. 24*, 6728-6741.

254. Palade, G. (1975) Intracellular aspects of the process of protein synthesis, *Science 189*, 347-358.

255. Pahl, H. L. (1999) Signal transduction from the endoplasmic reticulum to the cell nucleus, *Physiol. Rev. 79*, 683-701.

256. Borgese, N., Francolini, M., and Snapp, E. (2006) Endoplasmic reticulum architecture: structures in flux, *Curr. Opin. Cell Biol. 18*, 358-364.

257. Egea, P. F., Stroud, R. M., and Walter, P. (2005) Targeting proteins to membranes: structure of the signal recognition particle, *Curr. Opin. Struct. Biol. 15*, 213-220.

258. Keenan, R. J., Freymann, D. M., Stroud, R. M., and Walter, P. (2001) The signal recognition particle, *Annu. Rev. Biochem. 70*, 755-775.

259. Eichler, J., and Moll, R. (2001) The signal recognition particle of Archaea, *Trends Microbiol. 9*, 130-136.

260. Zopf, D., Bernstein, H. D., Johnson, A. E., and Walter, P. (1990) The methionine-rich domain of the 54 kd protein subunit of the signal recognition particle contains an RNA binding site and can be crosslinked to a signal sequence, *EMBO J. 9*, 4511-4517.

261. Lutcke, H., High, S., Romisch, K., Ashford, A. J., and Dobberstein, B. (1992) The methionine-rich domain of the 54 kDa subunit of signal recognition particle is sufficient for the interaction with signal sequences, *EMBO J. 11*, 1543-1551.

262. Keenan, R. J., Freymann, D. M., Walter, P., and Stroud, R. M. (1998) Crystal structure of the signal sequence binding subunit of the signal recognition particle, *Cell 94*, 181-191.

263. Brodsky, J. L. (1996) Post-translational protein translocation: not all hsc70s are created equal, *Trends Biochem. Sci. 21*, 122-126.

264. Stroud, R. M., and Walter, P. (1999) Signal sequence recognition and protein targeting, *Curr. Opin. Struct. Biol. 9*, 754-759.

265. Brodsky, J. L., Goeckeler, J., and Schekman, R. (1995) BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum, *PNAS USA 92*, 9643-9646.

266. Chirico, W. J., Waters, M. G., and Blobel, G. (1988) 70K heat shock related proteins stimulate protein translocation into microsomes, *Nature 332*, 805-810.

267. von Heijne, G. (1990) The signal peptide, *J. Membr. Biol. 115*, 195-201.

268. Doudna, J. A., and Batey, R. T. (2004) Structural insights into the signal recognition particle, *Annu. Rev. Biochem. 73*, 539-557.

269. Shan, S. O., and Walter, P. (2005) Co-translational protein targeting by the signal recognition particle, *FEBS Lett. 579*, 921-926.

270. Egea, P. F., Shan, S. O., Napetschnig, J., Savage, D. F., Walter, P., and Stroud, R. M. (2004) Substrate twinning activates the signal recognition particle and its receptor, *Nature 427*, 215-221.

271. Focia, P. J., Shepotinovskaya, I. V., Seidler, J. A., and Freymann, D. M. (2004) Heterodimeric GTPase core of the SRP targeting complex, *Science 303*, 373-377.

272. Menetret, J. F., Neuhof, A., Morgan, D. G., Plath, K., Radermacher, M., Rapoport, T. A., and Akey, C. W. (2000) The structure of ribosome-channel complexes engaged in protein translocation, *Mol. Cell 6*, 1219-1232.

273. Beckmann, R., Spahn, C. M., Eswar, N., Helmers, J., Penczek, P. A., Sali, A., Frank, J., and Blobel, G. (2001) Architecture of the protein-conducting channel associated with the translating 80S ribosome, *Cell 107*, 361-372.

274. High, S., and Dobberstein, B. (1992) Mechanisms that determine the transmembrane disposition of proteins, *Curr. Opin. Cell Biol. 4*, 581-586.

275. Higy, M., Junne, T., and Spiess, M. (2004) Topogenesis of membrane proteins at the endoplasmic reticulum, *Biochem. 43*, 12716-12722.

276. Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M., and Zimmermann, R. (1991) A microsomal protein is involved in ATP-dependent transport of presecretory proteins into mammalian microsomes, *EMBO J. 10*, 2795-2803.

277. Hansen, W., and Walter, P. (1988) Prepro-carboxypeptidase Y and a truncated form of pre-invertase, but not full-length pre-invertase, can be posttranslationally translocated across microsomal vesicle membranes from Saccharomyces cerevisiae, *J. Cell Biol. 106*, 1075-1081.

278. Schlenstedt, G., Gudmundsson, G. H., Boman, H. G., and Zimmermann, R. (1990) A large presecretory protein translocates both cotranslationally, using signal recognition particle and ribosome, and post-translationally, without these ribonucleoparticles, when synthesized in the presence of mammalian microsomes, *J. Biol. Chem. 265*, 13960-13968.

279. Schlenstedt, G., Gudmundsson, G. H., Boman, H. G., and Zimmermann, R. (1992) Structural requirements for transport of preprocecropinA and related presecretory proteins into mammalian microsomes, *J. Biol. Chem. 267*, 24328-24332.

280. Zimmermann, R., Sagstetter, M., Lewis, M. J., and Pelham, H. R. (1988) Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes, *EMBO J. 7*, 2875-2880.

281. Wilkinson, B. M., Regnacq, M., and Stirling, C. J. (1997) Protein translocation across the membrane of the endoplasmic reticulum, *J. Membr. Biol. 155*, 189-197.

282. Wiech, H., Stuart, R., and Zimmermann, R. (1990) Role of cytosolic factors in the transport of proteins across membranes, *Semin. Cell Biol. 1*, 55-63.

283. Martoglio, B., and Dobberstein, B. (1998) Signal sequences: more than just greasy peptides, *Trends Cell Biol. 8*, 410-415.

284. Hegde, R. S., and Bernstein, H. D. (2006) The surprising complexity of signal sequences, *Trends Biochem. Sci. 31*, 563-571.

285. Rutkowski, D. T., Lingappa, V. R., and Hegde, R. S. (2001) Substrate-specific regulation of the ribosome- translocon junction by N-terminal signal sequences, *PNAS USA 98*, 7823-7828.

286. Kim, S. J., Mitra, D., Salerno, J. R., and Hegde, R. S. (2002) Signal sequences control gating of the protein translocation channel in a substrate-specific manner, *Dev. Cell 2*, 207-217.

287. Voigt, S., Jungnickel, B., Hartmann, E., and Rapoport, T. A. (1996) Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum membrane, *J. Cell Biol. 134*, 25-35.

288. Fons, R. D., Bogert, B. A., and Hegde, R. S. (2003) Substrate-specific function of the translocon-associated protein complex during translocation across the ER membrane, *J. Cell Biol. 160*, 529-539.

289. Belin, D., Bost, S., Vassalli, J. D., and Strub, K. (1996) A two-step recognition of signal sequences determines the translocation efficiency of proteins, *EMBO J. 15*, 468-478.

290. Levine, C. G., Mitra, D., Sharma, A., Smith, C. L., and Hegde, R. S. (2005) The efficiency of protein compartmentalization into the secretory pathway, *Mol. Biol. Cell 16*, 279-291.

291. Shaffer, K. L., Sharma, A., Snapp, E. L., and Hegde, R. S. (2005) Regulation of protein compartmentalization expands the diversity of protein function, *Dev. Cell 9*, 545-554.

292. Besemer, J., Harant, H., Wang, S., Oberhauser, B., Marquardt, K., Foster, C. A., Schreiner, E. P., de Vries, J. E., Dascher-Nadel, C., and Lindley, I. J. (2005) Selective inhibition of cotranslational translocation of vascular cell adhesion molecule 1, *Nature 436*, 290-293.

293. Garrison, J. L., Kunkel, E. J., Hegde, R. S., and Taunton, J. (2005) A substrate-specific inhibitor of protein translocation into the endoplasmic reticulum, *Nature 436*, 285-289.

294. Kelly, J. W., and Balch, W. E. (2006) The integration of cell and chemical biology in protein folding, *Nat. Chem. Biol. 2*, 224-227.

295. Aridor, M. (2007) Visiting the ER: the endoplasmic reticulum as a target for therapeutics in traffic related diseases, *Adv. Drug Deliv. Rev. 59*, 759-781.

296. Kopito, R. R. (1999) Biosynthesis and degradation of CFTR, *Physiol. Rev. 79*, S167-173.

297. Peters, K. W., Qi, J., Watkins, S. C., and Frizzell, R. A. (2000) Mechanisms underlying regulated CFTR trafficking, *Med. Clin. North Am. 84*, 633-640, ix-x.

298. Brennan, S. O., Wyatt, J., Medicina, D., Callea, F., and George, P. M. (2000) Fibrinogen brescia: hepatic endoplasmic reticulum storage and hypofibrinogenemia because of a gamma284 Gly-->Arg mutation, *Am. J. Pathol. 157*, 189-196.

299. Niwa, M., Sidrauski, C., Kaufman, R. J., and Walter, P. (1999) A role for presenilin-1 in nuclear accumulation of Ire1 fragments and induction of the mammalian unfolded protein response, *Cell 99*, 691-702.

300. Nogalska, A., Engel, W. K., McFerrin, J., Kokame, K., Komano, H., and Askanas, V. (2006) Homocysteine-induced endoplasmic reticulum protein (Herp) is up-regulated in sporadic inclusion-body myositis and in endoplasmic reticulum stress-induced cultured human muscle fibers, *J. Neurochem. 96*, 1491-1499.

301. Sai, X., Kawamura, Y., Kokame, K., Yamaguchi, H., Shiraishi, H., Suzuki, R., Suzuki, T., Kawaichi, M., Miyata, T., Kitamura, T., De Strooper, B., Yanagisawa, K., and Komano, H. (2002) Endoplasmic reticulum stress-inducible protein, Herp, enhances presenilin-mediated generation of amyloid beta-protein, *J. Biol. Chem. 277*, 12915-12920.

302. Cooper, A. A., Gitler, A. D., Cashikar, A., Haynes, C. M., Hill, K. J., Bhullar, B., Liu, K., Xu, K., Strathearn, K. E., Liu, F., Cao, S., Caldwell, K. A., Caldwell, G. A., Marsischky, G., Kolodner, R. D., Labaer, J., Rochet, J. C., Bonini, N. M., and Lindquist, S. (2006) Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models, *Science 313*, 324-328.

303. Taylor, S. I. (1992) Lilly Lecture: molecular mechanisms of insulin resistance. Lessons from patients with mutations in the insulin-receptor gene, *Diabetes 41*, 1473-1490.

304. Kadowaki, T., Kadowaki, H., Accili, D., Yazaki, Y., and Taylor, S. I. (1991) Substitution of arginine for histidine at position 209 in the alpha-subunit of the human insulin receptor. A mutation that impairs receptor dimerization and transport of receptors to the cell surface, *J. Biol. Chem. 266*, 21224-21231.

305. Goda, Y., and Pfeffer, S. R. (1988) Selective recycling of the mannose 6-phosphate/IGF-II receptor to the trans Golgi network in vitro, *Cell 55*, 309-320.

306. Ghosh, P., Dahms, N. M., and Kornfeld, S. (2003) Mannose 6-phosphate receptors: new twists in the tale, *Nat. Rev. Mol. Cell Biol. 4*, 202-212.

307. Duncan, J. R., and Kornfeld, S. (1988) Intracellular movement of two mannose 6-phosphate receptors: return to the Golgi apparatus, *J. Cell Biol. 106*, 617-628.

308. Rane, N. S., Chakrabarti, O., Feigenbaum, L., and Hegde, R. S. (2010) Signal sequence insufficiency contributes to neurodegeneration caused by transmembrane prion protein, *J. Cell Biol. 188*, 515-526.

309. Angelotti, T., Daunt, D., Shcherbakova, O. G., Kobilka, B., and Hurt, C. M. (2010) Regulation of G-Protein Coupled Receptor Traffic by an Evolutionary Conserved Hydrophobic Signal, *Traffic. 11*, 560-578.

310. Futatsumori-Sugai, M., and Tsumoto, K. (2010) Signal peptide design for improving recombinant protein secretion in the baculovirus expression vector system, *Biochem. Biophys. Res. Commun. 391*, 931-935.

311. Loera-Arias, M. J., Martinez-Perez, A. G., Barrera-Hernandez, A., Ibarra-Obregon, E. R., Gonzalez-Saldivar, G., Martinez-Ortega, J. I., Rosas-Taraco, A., Villanueva-Olivo, A., Esparza-Gonzalez, S. C., Villatoro-Hernandez, J., Saucedo-Cardenas, O., and Montes-de-Oca-Luna, R. (2009) Targeting and retention of HPV16 E7 to the endoplasmic reticulum enhances immune tumor protection, *J. Cell Mol. Med. 14*, 890-894.

312. Snyder, H. L., Yewdell, J. W., and Bennink, J. R. (1994) Trimming of antigenic peptides in an early secretory compartment, *J. Exp. Med. 180*, 2389-2394.

313. Levelt, C. N., de Jong, Y. P., Mizoguchi, E., O'Farrelly, C., Bhan, A. K., Tonegawa, S., Terhorst, C., and Simpson, S. J. (1999) High- and low-affinity single-peptide/MHC ligands have distinct effects on the development of mucosal CD8alphaalpha and CD8alphabeta T lymphocytes, *PNAS USA 96*, 5628-5633.

314. Krajcsi, P., Tollefson, A. E., Anderson, C. W., and Wold, W. S. (1992) The adenovirus E3 14.5-kilodalton protein, which is required for down-regulation of the epidermal growth factor receptor and prevention of tumor necrosis factor cytolysis, is an integral membrane protein oriented with its C terminus in the cytoplasm, *J. Virol. 66*, 1665-1673.

315. Zou, Z., and Sun, P. D. (2006) An improved recombinant mammalian cell expression system for human transforming growth factor-beta2 and -beta3 preparations, *Protein Expr. Purif. 50*, 9-17.

316. Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988) The mannose 6-phosphate receptor and the biogenesis of lysosomes, *Cell 52*, 329-341.

317. Geuze, H. J., Stoorvogel, W., Strous, G. J., Slot, J. W., Bleekemolen, J. E., and Mellman, I. (1988) Sorting of mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles, *J. Cell Biol. 107*, 2491-2501.

318. Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B., and Johannes, L. (1998) Direct pathway from early/recycling endosomes to the Golgi apparatus

revealed through the study of shiga toxin B-fragment transport, *J. Cell Biol. 143*, 973-990.

319. Schapiro, F. B., Lingwood, C., Furuya, W., and Grinstein, S. (1998) pH-independent retrograde targeting of glycolipids to the Golgi complex, *Am. J. Physiol. 274*, C319-332.

320. Lauvrak, S. U., Torgersen, M. L., and Sandvig, K. (2004) Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin, *J. Cell Sci. 117*, 2321-2331.

321. Iversen, T. G., Skretting, G., Llorente, A., Nicoziani, P., van Deurs, B., and Sandvig, K. (2001) Endosome to Golgi transport of ricin is independent of clathrin and of the Rab9- and Rab11-GTPases, *Mol. Biol. Cell 12*, 2099-2107.

322. Grimmer, S., Iversen, T. G., van Deurs, B., and Sandvig, K. (2000) Endosome to Golgi transport of ricin is regulated by cholesterol, *Mol. Biol. Cell 11*, 4205-4216.

323. Stanley, K. K., and Howell, K. E. (1993) TGN38/41: a molecule on the move, *Trends Cell Biol. 3*, 252-255.

324. Molloy, S. S., Anderson, E. D., Jean, F., and Thomas, G. (1999) Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis, *Trends Cell Biol. 9*, 28-35.

325. Varlamov, O., and Fricker, L. D. (1998) Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface, *J. Cell Sci. 111 (Pt 7)*, 877-885.

326. Bos, K., Wraight, C., and Stanley, K. K. (1993) TGN38 is maintained in the trans-Golgi network by a tyrosine-containing motif in the cytoplasmic domain, *EMBO J. 12*, 2219-2228.

327. Schafer, W., Stroh, A., Berghofer, S., Seiler, J., Vey, M., Kruse, M. L., Kern, H. F., Klenk, H. D., and Garten, W. (1995) Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin, *EMBO J. 14*, 2424-2435.

328. Voorhees, P., Deignan, E., van Donselaar, E., Humphrey, J., Marks, M. S., Peters, P. J., and Bonifacino, J. S. (1995) An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface, *EMBO J. 14*, 4961-4975.

329. Molloy, S. S., Thomas, L., Kamibayashi, C., Mumby, M. C., and Thomas, G. (1998) Regulation of endosome sorting by a specific PP2A isoform, *J. Cell Biol. 142*, 1399-1411.

330. Dittie, A. S., Thomas, L., Thomas, G., and Tooze, S. A. (1997) Interaction of furin in immature secretory granules from neuroendocrine cells with the AP-1 adaptor complex is modulated by casein kinase II phosphorylation, *EMBO J. 16*, 4859-4870.

331. Alconada, A., Bauer, U., and Hoflack, B. (1996) A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the trans-Golgi network, *EMBO J. 15*, 6096-6110.

332. Smith, D. C., Lord, J. M., Roberts, L. M., Tartour, E., and Johannes, L. (2002) 1st class ticket to class I: protein toxins as pathfinders for antigen presentation, *Traffic 3*, 697-704.

333. Lee, R. S., Tartour, E., van der Bruggen, P., Vantomme, V., Joyeux, I., Goud, B., Fridman, W. H., and Johannes, L. (1998) Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of Shiga toxin, *Eur. J. Immunol. 28*, 2726-2737.

334. Haicheur, N., Bismuth, E., Bosset, S., Adotevi, O., Warnier, G., Lacabanne, V., Regnault, A., Desaymard, C., Amigorena, S., Ricciardi-Castagnoli, P., Goud, B., Fridman, W. H., Johannes, L., and Tartour, E. (2000) The B subunit of Shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class I-restricted presentation of peptides derived from exogenous antigens, *J. Immunol. 165*, 3301-3308.

335. Haicheur, N., Benchetrit, F., Amessou, M., Leclerc, C., Falguieres, T., Fayolle, C., Bismuth, E., Fridman, W. H., Johannes, L., and Tartour, E. (2003) The B subunit of Shiga toxin coupled to full-size antigenic protein elicits humoral and cell-mediated immune responses associated with a Th1-dominant polarization, *Int. Immunol. 15*, 1161-1171.

336. Vingert, B., Adotevi, O., Patin, D., Jung, S., Shrikant, P., Freyburger, L., Eppolito, C., Sapoznikov, A., Amessou, M., Quintin-Colonna, F., Fridman, W. H., Johannes, L., and Tartour, E. (2006) The Shiga toxin B-subunit targets antigen in vivo to dendritic cells and elicits anti-tumor immunity, *Eur. J. Immunol. 36*, 1124-1135.

337. Janssen, K. P., Vignjevic, D., Boisgard, R., Falguieres, T., Bousquet, G., Decaudin, D., Dolle, F., Louvard, D., Tavitian, B., Robine, S., and Johannes, L. (2006) In vivo tumor targeting using a novel intestinal pathogen-based delivery approach, *Cancer Res. 66*, 7230-7236.

338. Kovbasnjuk, O., Mourtazina, R., Baibakov, B., Wang, T., Elowsky, C., Choti, M. A., Kane, A., and Donowitz, M. (2005) The glycosphingolipid

globotriaosylceramide in the metastatic transformation of colon cancer, *PNAS USA 102*, 19087-19092.

339. Arab, S., Russel, E., Chapman, W. B., Rosen, B., and Lingwood, C. A. (1997) Expression of the verotoxin receptor glycolipid, globotriaosylceramide, in ovarian hyperplasias, *Oncol. Res. 9*, 553-563.

340. LaCasse, E. C., Bray, M. R., Patterson, B., Lim, W. M., Perampalam, S., Radvanyi, L. G., Keating, A., Stewart, A. K., Buckstein, R., Sandhu, J. S., Miller, N., Banerjee, D., Singh, D., Belch, A. R., Pilarski, L. M., and Gariepy, J. (1999) Shiga-like toxin-1 receptor on human breast cancer, lymphoma, and myeloma and absence from CD34(+) hematopoietic stem cells: implications for ex vivo tumor purging and autologous stem cell transplantation, *Blood 94*, 2901-2910.

341. LaCasse, E. C., Saleh, M. T., Patterson, B., Minden, M. D., and Gariepy, J. (1996) Shiga-like toxin purges human lymphoma from bone marrow of severe combined immunodeficient mice, *Blood 88*, 1561-1567.

342. Arab, S., Murakami, M., Dirks, P., Boyd, B., Hubbard, S. L., Lingwood, C. A., and Rutka, J. T. (1998) Verotoxins inhibit the growth of and induce apoptosis in human astrocytoma cells, *J. Neurooncol. 40*, 137-150.

343. Salhia, B., Rutka, J. T., Lingwood, C., Nutikka, A., and Van Furth, W. R. (2002) The treatment of malignant meningioma with verotoxin, *Neoplasia 4*, 304-311.

344. Hicks, S. W., and Machamer, C. E. (2005) Golgi structure in stress sensing and apoptosis, *Biochim. Biophys. Acta. 1744*, 406-414.

345. De Duve, C., and Baudhuin, P. (1966) Peroxisomes (microbodies and related particles), *Physiol. Rev. 46*, 323-357.

346. Jansen, G. A., and Wanders, R. J. (2006) Alpha-oxidation, *Biochim. Biophys. Acta. 1763*, 1403-1412.

347. Heiland, I., and Erdmann, R. (2005) Biogenesis of peroxisomes. Topogenesis of the peroxisomal membrane and matrix proteins, *FEBS J. 272*, 2362-2372.

348. Gould, S. G., Keller, G. A., and Subramani, S. (1987) Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase, *J. Cell Biol. 105*, 2923-2931.

349. Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J., and Subramani, S. (1989) A conserved tripeptide sorts proteins to peroxisomes, *J. Cell Biol. 108*, 1657-1664.

350. Lametschwandtner, G., Brocard, C., Fransen, M., Van Veldhoven, P., Berger, J., and Hartig, A. (1998) The difference in recognition of terminal tripeptides as

peroxisomal targeting signal 1 between yeast and human is due to different affinities of their receptor Pex5p to the cognate signal and to residues adjacent to it, *J. Biol. Chem. 273*, 33635-33643.

351. Brocard, C., and Hartig, A. (2006) Peroxisome targeting signal 1: is it really a simple tripeptide?, *Biochim. Biophys. Acta. 1763*, 1565-1573.

352. Bongcam, V., MacDonald-Comber Petetot, J., Mittendorf, V., Robertson, E. J., Leech, R. M., Qin, Y. M., Hiltunen, J. K., and Poirier, Y. (2000) Importance of sequences adjacent to the terminal tripeptide in the import of a peroxisomal Candida tropicalis protein in plant peroxisomes, *Planta 211*, 150-157.

353. Marzioch, M., Erdmann, R., Veenhuis, M., and Kunau, W. H. (1994) PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes, *EMBO J. 13*, 4908-4918.

354. Reumann, S. (2004) Specification of the peroxisome targeting signals type 1 and type 2 of plant peroxisomes by bioinformatics analyses, *Plant Physiol. 135*, 783-800.

355. Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A., and Subramani, S. (1991) A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase, *EMBO J. 10*, 3255-3262.

356. Wanders, R. J., and Waterham, H. R. (2005) Peroxisomal disorders I: biochemistry and genetics of peroxisome biogenesis disorders, *Clin. Genet. 67*, 107-133.

357. McCollum, D., Monosov, E., and Subramani, S. (1993) The pas8 mutant of Pichia pastoris exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells--the PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family, *J. Cell Biol. 121*, 761-774.

358. Terlecky, S. R., Nuttley, W. M., McCollum, D., Sock, E., and Subramani, S. (1995) The Pichia pastoris peroxisomal protein PAS8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal, *EMBO J. 14*, 3627-3634.

359. Keller, G. A., Gould, S., Deluca, M., and Subramani, S. (1987) Firefly luciferase is targeted to peroxisomes in mammalian cells, *PNAS USA 84*, 3264-3268.

360. Goth, L. (2000) Lipid and carbohydrate metabolism in acatalasemia, *Clin. Chem. 46*, 564-566.

361. Fransen, M., Terlecky, S. R., and Subramani, S. (1998) Identification of a human PTS1 receptor docking protein directly required for peroxisomal protein import, *PNAS USA 95*, 8087-8092.

362. Rayapuram, N., and Subramani, S. (2006) The importomer--a peroxisomal membrane complex involved in protein translocation into the peroxisome matrix, *Biochim. Biophys. Acta. 1763*, 1613-1619.

363. Leon, S., Goodman, J. M., and Subramani, S. (2006) Uniqueness of the mechanism of protein import into the peroxisome matrix: transport of folded, co-factor-bound and oligomeric proteins by shuttling receptors, *Biochim. Biophys. Acta. 1763*, 1552-1564.

364. Dodt, G., and Gould, S. J. (1996) Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor, *J. Cell Biol. 135*, 1763-1774.

365. van der Klei, I. J., and Veenhuis, M. (1996) Peroxisome biogenesis in the yeast Hansenula polymorpha: a structural and functional analysis, *Ann. NY Acad. Sci. 804*, 47-59.

366. Nair, D. M., Purdue, P. E., and Lazarow, P. B. (2004) Pex7p translocates in and out of peroxisomes in Saccharomyces cerevisiae, *J. Cell Biol. 167*, 599-604.

367. Dammai, V., and Subramani, S. (2001) The human peroxisomal targeting signal receptor, Pex5p, is translocated into the peroxisomal matrix and recycled to the cytosol, *Cell 105*, 187-196.

368. Dyer, J. M., McNew, J. A., and Goodman, J. M. (1996) The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop, *J. Cell Biol. 133*, 269-280.

369. Pause, B., Saffrich, R., Hunziker, A., Ansorge, W., and Just, W. W. (2000) Targeting of the 22 kDa integral peroxisomal membrane protein, *FEBS Lett. 471*, 23-28.

370. Honsho, M., and Fujiki, Y. (2001) Topogenesis of peroxisomal membrane protein requires a short, positively charged intervening-loop sequence and flanking hydrophobic segments. study using human membrane protein PMP34, *J. Biol. Chem. 276*, 9375-9382.

371. Honsho, M., Hiroshige, T., and Fujiki, Y. (2002) The membrane biogenesis peroxin Pex16p. Topogenesis and functional roles in peroxisomal membrane assembly, *J. Biol. Chem. 277*, 44513-44524.

372. Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P., and Tabak, H. F. (2005) Contribution of the endoplasmic reticulum to peroxisome formation, *Cell 122*, 85-95.

373. Voorn-Brouwer, T., Kragt, A., Tabak, H. F., and Distel, B. (2001) Peroxisomal membrane proteins are properly targeted to peroxisomes in the absence of COPI- and COPII-mediated vesicular transport, *J. Cell Sci. 114*, 2199-2204.

374. South, S. T., Sacksteder, K. A., Li, X., Liu, Y., and Gould, S. J. (2000) Inhibitors of COPI and COPII do not block PEX3-mediated peroxisome synthesis, *J. Cell Biol. 149*, 1345-1360.

375. Terlecky, S. R., and Koepke, J. I. (2007) Drug delivery to peroxisomes: employing unique trafficking mechanisms to target protein therapeutics, *Adv. Drug Deliv. Rev. 59*, 739-747.

376. Wood, C. S., Koepke, J. I., Teng, H., Boucher, K. K., Katz, S., Chang, P., Terlecky, L. J., Papanayotou, I., Walton, P. A., and Terlecky, S. R. (2006) Hypocatalasemic fibroblasts accumulate hydrogen peroxide and display age-associated pathologies, *Traffic 7*, 97-107.

377. Koepke, J. I., Nakrieko, K. A., Wood, C. S., Boucher, K. K., Terlecky, L. J., Walton, P. A., and Terlecky, S. R. (2007) Restoration of peroxisomal catalase import in a model of human cellular aging, *Traffic 8*, 1590-1600.

378. Lisenbee, C. S., Lingard, M. J., and Trelease, R. N. (2005) Arabidopsis peroxisomes possess functionally redundant membrane and matrix isoforms of monodehydroascorbate reductase, *Plant J. 43*, 900-914.

379. Mizuno, T., Ito, K., Uchida, C., Kitagawa, M., Ichiyama, A., Miura, S., Fujita, K., and Oda, T. (2002) Analyses in transfected cells and in vitro of a putative peroxisomal targeting signal of rat liver serine:pyruvate aminotransferase, *Histochem. Cell Biol. 118*, 321-328.

380. Horiguchi, H., Yurimoto, H., Goh, T., Nakagawa, T., Kato, N., and Sakai, Y. (2001) Peroxisomal catalase in the methylotrophic yeast Candida boidinii: transport efficiency and metabolic significance, *J. Bacteriol. 183*, 6372-6383.

381. Nalepa, G., Rolfe, M., and Harper, J. W. (2006) Drug discovery in the ubiquitin-proteasome system, *Nat. Rev. 5*, 596-613.

382. Dahlmann, B. (2007) Role of proteasomes in disease, *BMC Biochem. 8 Suppl 1*, S3.

383. Elliott, P. J., and Ross, J. S. (2001) The proteasome: a new target for novel drug therapies, *Am. J Clin. Pathol. 116*, 637-646.

384. Richardson, P. G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S. V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orlowski, R. Z., Kuter, D., Limentani, S. A., Lee, S., Hideshima, T., Esseltine, D. L., Kauffman, M., Adams, J., Schenkein, D. P., and Anderson, K. C. (2003) A phase 2 study of bortezomib in relapsed, refractory myeloma, *N. Engl. J. Med. 348*, 2609-2617.

385. Kyle, R. A., and Rajkumar, S. V. (2004) Multiple myeloma, *N. Engl. J. Med. 351*, 1860-1873.

386. Adams, J. (2004) The development of proteasome inhibitors as anticancer drugs, *Cancer Cell 5*, 417-421.

387. Burger, A. M., and Seth, A. K. (2004) The ubiquitin-mediated protein degradation pathway in cancer: therapeutic implications, *Eur. J. Cancer 40*, 2217-2229.

388. Robinson, P. A., and Ardley, H. C. (2004) Ubiquitin-protein ligases--novel therapeutic targets?, *Curr. Protein Peptide Sci. 5*, 163-176.

389. Honda, R., and Yasuda, H. (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53, *EMBO J. 18*, 22-27.

390. Lowe, S. W., and Sherr, C. J. (2003) Tumor suppression by Ink4a-Arf: progress and puzzles, *Curr. Opin. Genet. Dev. 13*, 77-83.

391. Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops, *Oncogene 24*, 2899-2908.

392. Wagner, E. (2004) Strategies to improve DNA polyplexes for in vivo gene transfer: will "artificial viruses" be the answer?, *Pharm. Res. 21*, 8-14.

CHAPTER 3

REVERSAL OF OXIDATIVE STRESS IN ENDOTHELIAL CELLS BY CONTROLLED RELEASE OF ADIPONECTIN

Abstract

Hyperglycemia causes endothelial dysfunction due to its effect on increasing reactive oxygen species (ROS). Adiponectin (Adp) has been reported to suppress hyperglycemia-associated ROS-generation. It was hypothesized that administering globular adiponectin (gAdp) via injectable biodegradable thermosensitive triblock copolymer might effectively reduce ROS generation in endothelial cells. In this study, gAdp was incorporated into and released from the polymer gel. The released gAdp was further investigated by comparing it with the intact gAdp with regard to the efficiency in reducing ROS and activating cAMP. The released gAdp effectively suppressed excess ROS-production in the *in vitro* endothelial cell culture model under high-glucose condition via cAMP/PKA pathway. These data provide a rationale for developing controlled release dosage form of gAdp as a therapeutic tool for oxidative stress-related pathology in patients with diabetes.

Reprinted by permission from the *Journal of Controlled Release* 130: 234-7 (2008).
Mossalam M., Jeong J., Abel E.D., Kim S.W., Kim Y.
M.M. performed the experiments and wrote the first draft. The rest of authors contributed with scientific discussion and feedback.

Introduction

Hyperglycemia is one of the main causes of vascular complications in diabetic patients due to its effect on increasing ROS-production, which causes endothelial dysfunction (*1-3*). In blood vessels, ROS have been implicated in the pathogenesis of hypertension, vascular lesion formation, atherosclerosis, and diabetic vascular disease (*4-8*). Consequently, a novel approach to restore vascular function in diabetes might be to reduce mitochondrial ROS-overproduction.

Adiponectin (Adp) is a circulating hormone secreted by adipose tissue that plays a key role in glucose homeostasis and fatty acid metabolism (*9*). Adp levels are reduced in patients with obesity and cardiovascular disease (*10, 11*). Studies have shown the potential role of Adp in coronary artery disease patients with diabetes by suppressing ROS generation (*12-15*). Adp has also been very recently recognized to suppress hyperglycemia-associated ROS-generation via a cAMP-dependent protein kinase (PKA) pathway (*16*).

Adiponectin could be administered using an injectable biodegradable thermosensitive triblock copolymer delivery system. The copolymer, which is composed of hydrophilic poly (ethylene glycol) (PEG) B-block and hydrophobic poly (lactide-co-glycolide) (PLGA) A block, allows the delivery of insoluble or unstable substances by generating a hydrogel depot at body temperature (*17, 18*). It has also been used to enhance gene delivery efficiency (*19*). Potentially, these copolymers were proved to be valuable components of controlled delivery systems or site-specific administration of therapeutics (*20, 21*).

The aim of this study was to develop and characterize a controlled drug delivery system containing gAdp, and test the hypothesis that administering gAdp via a polymer gel might effectively reduce ROS-generation in endothelial cells and therefore lead to recovery of endothelial dysfunction. We investigated the antioxidant effect and mechanism of action of released gAdp by the copolymer on reducing ROS in Bovine Aortic Endothelial Cells (BAEC). This study investigates a new therapeutical advantage in gAdp delivery and also provides the framework for further work involving *in vivo* studies.

Materials and Methods

BAECs were obtained from Cambrex Bio Science Walkersville, Inc. Cell culture materials were from Invitrogen (Carlsbad, CA). The copolymer (ReGel® R40/60) was kindly provided by MacroMed, Inc (Salt Lake City, UT), Chen S., et al. has detailed synthesis and characterization of the copolymer (*22*). The mouse recombinant gAdp (16.6 kDa in E. coli host) and fAdp (35kDa in HEK 293 cells host) were from Kamiya Biomedical Company (Seattle, WA). All other chemicals and reagents, unless otherwise noted, were obtained from Sigma (St. Louis, MO).

Cell Culture

BAECs were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Cells were routinely studied before the tenth passage and at 80-90% confluence for ROS and cAMP measurements.

In Vitro ROS Measurements

BAECs at a density of 2×10^5 cells/well in 6 well plates were treated under different glucose concentrations: 0 mM (as a negative control), 15 mM, and 25 mM (as a positive control). The 25 mM glucose treated cells were then treated with 2 μg/ml, 3μg/ml, 4.5μg/ml, 7 μg/ml and 9μg/ml globular adiponectin (gAdp), and 4.5 μg/ml released globular adiponectin from the polymer gel (rAdp) to measure the effect of adiponectin on the hyperglycemic-induced ROS. The cells were cultured for 36 hours, then treated with 10 μM 5-(and-6)-chloromethyl-2`,7`-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) for 30 minutes. They were then suspended in Phosphate-Buffered Saline (PBS). The fluorescence was then measured using flow cytometry. The higher the fluorescence, the greater the ROS production (*23*).

Measurements of Cellular cAMP

BAECs at a density of 2×10^5 cells/well were cultured under the same conditions as in the *in vitro* ROS studies. cAMP was then measured by following the manufacturer's instruction using a direct immunoassay kit (BioVision) (*24*).

In Vitro Release Study of gAdp from the Copolymer

Firstly, 25 μg gAdp were dissolved in 0.5 ml the copolymer and incubated in a 37°C water bath. Once gel is formed, 1 ml of PBS was added to and removed from the vial containing the polymer gel every 24 hours. The released protein was quantified using Adiponectin ELISA kit (B-Bridge International) and then concentrated at different time

intervals using centriprep (Amicon) to further investigate its activity. SDS was performed on the concentrated protein (*25*).

Statistical Analyses

Quantitative data are presented as means ± SD as indicated from the number of experiments. Statistical analysis was based on Student's t-test for comparison of two groups. A *p* value less than 0.05 was used to determine statistical significance.

Results and Discussion

The method used to measure ROS-overproduction was confirmed by testing the effect of gAdp on reducing ROS. BAECs were incubated in different concentrations of glucose. There was an increase in the ROS-production with the glucose treated cells in a dose dependent manner up to 7.5 fold ($P < 0.05$) (Figure 3.1). However, when using mannitol at the same concentrations, there was no significant difference in the ROS ruling out any osmotic effect. Hyperglycemia induces ROS-overproduction through several pathways. A major mechanism is through the overproduction of the superoxide anion (O^{-2}) by the mitochondrial electron transport chain (*26*).

Incubating the cells in 25 mM glucose medium puts the cells in hyperglycemic condition. The high-glucose treated cells followed by the incubation with gAdp showed reduction in ROS-production in a dose dependent manner with optimum effect at 4.5 μg/ml concentration (Figure 3.2). It had no effect on the no-glucose treated cells showing its activity merely on the ROS induced by glucose treatment.

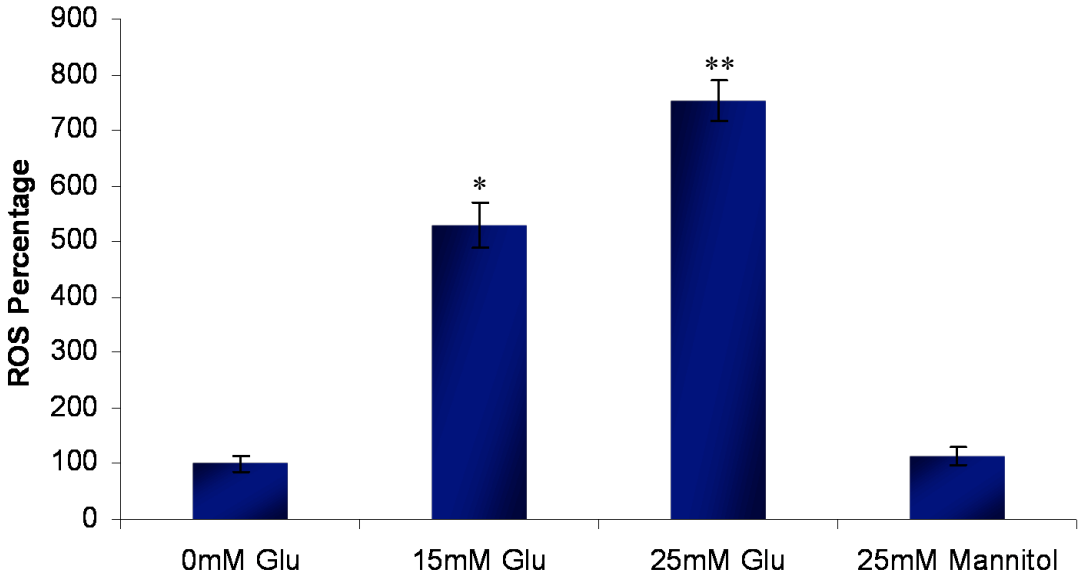


Figure 3.1. Glucose effect on ROS production. After treatment with glucose, ROS was measured via FACSCAN. Mannitol had no effect on ROS. Data are expressed as the percent of basal (0mM glucose) and represent the means ± SD of n=3. *P<0.01 and **P<0.001 vs. basal. Statistical analysis was corrected after publication. A one-way ANOVA was performed with Bonferroni's multiple comparison test.

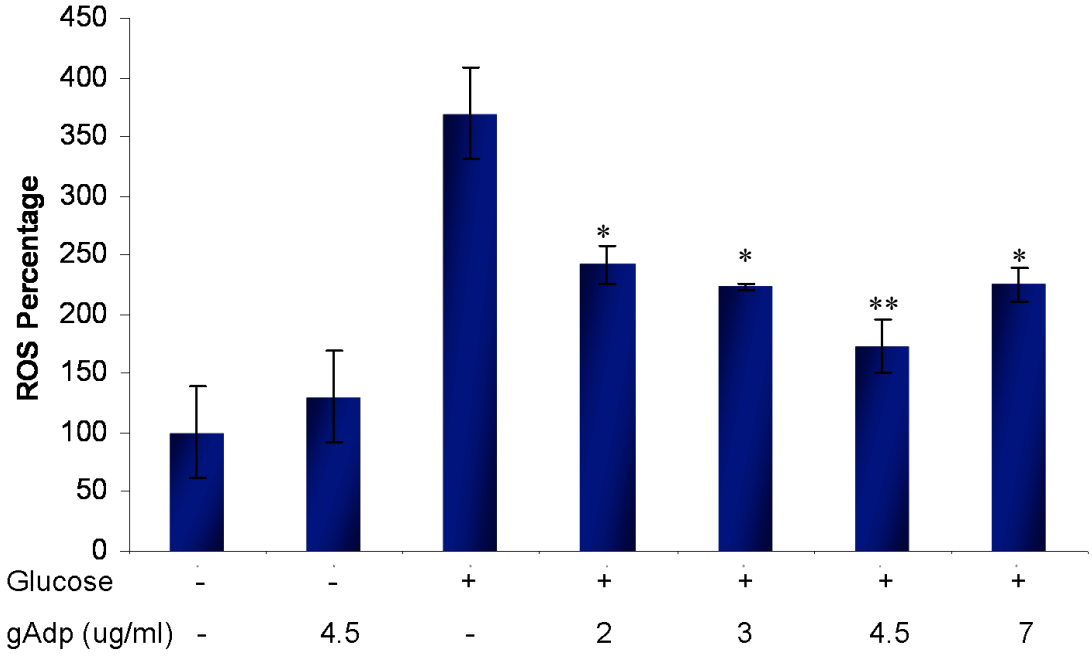


Figure 3.2. The gAdp dose response in reducing ROS, induced by high glucose in endothelial cells. After treatment, ROS was measured via FACSCAN. The gAdp showed no effect on 0 mM treated cells. Data are expressed as the percent of basal (0mM glucose) and represent the means ± SD of n=3. *P<0.01 and **P<0.001 vs. glucose treated cells. Statistical analysis was corrected after publication. A two-way ANOVA was performed with Bonferroni's posttest.

The controlled delivery system containing gAdp was developed by solubilizing the protein in the polymer gel. *In vitro* release of gAdp from the polymer gel and its activity on ROS and cAMP were tested to characterize and prove the efficacy of the delivery model. The release profile showed almost 100% release of the 25 μg gAdp (Figure 3.3) over 27 days without initial burst release. The protein was released in a first order manner in the first 5 days, then in a zero-order manner in the following 10 days, and then in a zero-order manner with a different release rate in the final 12 days. The protein is released from the gel upon degradation of the copolymer. The released protein was then concentrated using centriprep (Amicon) to further investigate its activity. The SDS PAGE performed on the released Adp at different time intervals did not show any degradation or aggregation, which shows the stability of the released protein from the polymer gel (Figure 3.4).

The released gAdp (rAdp) from the copolymer was compared to the activity of the intact gAdp. It showed that the released gAdp had the same effect as the intact gAdp on reducing ROS in endothelial cells with no significant difference (Figure 3.5). These results represent the potential of the triblock copolymer as a safe delivery carrier for the gAdp.

cAMP was measured in high glucose induced cells treated with either gAdp or rAdp. The gAdp and rAdp showed the same effect on increasing the cellular content of cAMP with no significant difference (Figure 3.6) proving the pathway in which gAdp reduces ROS and the preservation of the activity of the released gAdp. The rAdp had no change in its characteristics in relation to its activity on ROS and cAMP.

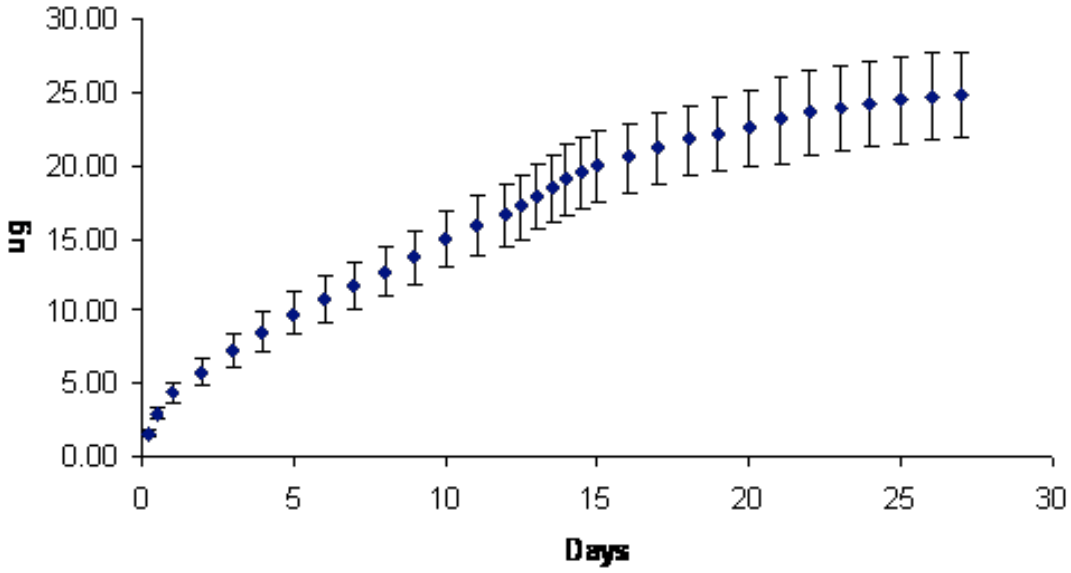


Figure 3.3. *In vitro* release study of 25 µg gAdp from injectable, biodegradable, and thermosensitive triblock copolymer. The gAdp is fully released over the 27 days. The data represent means ± SD of n=5.

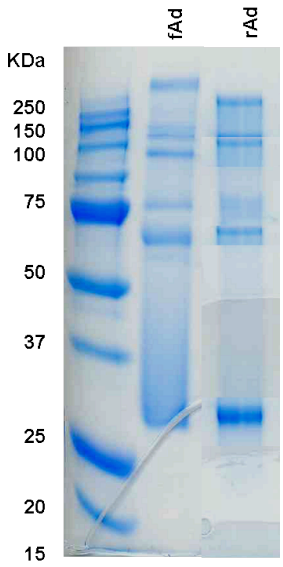


Figure 3.4. SDS PAGE for the released adiponectin compared to the intact adiponectin. It shows no degradation of the protein upon release from the polymer gel.

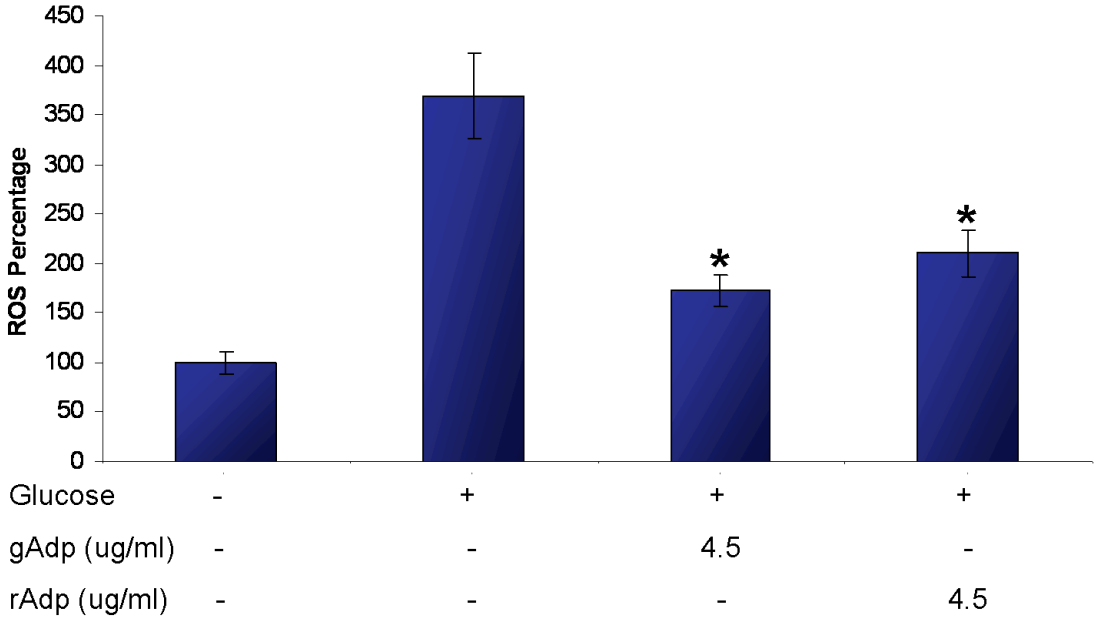


Figure 3.5. Effect of the released gAdp (rAdp) from the polymer gel on reducing ROS compared to the intact gAdp. Data are expressed as the percent of basal (0mM glucose) and represent the means ± SD of n=3. *P<0.05 vs. high glucose. Statistical analysis was corrected after publication. A two-way ANOVA was performed with Bonferroni's posttest.

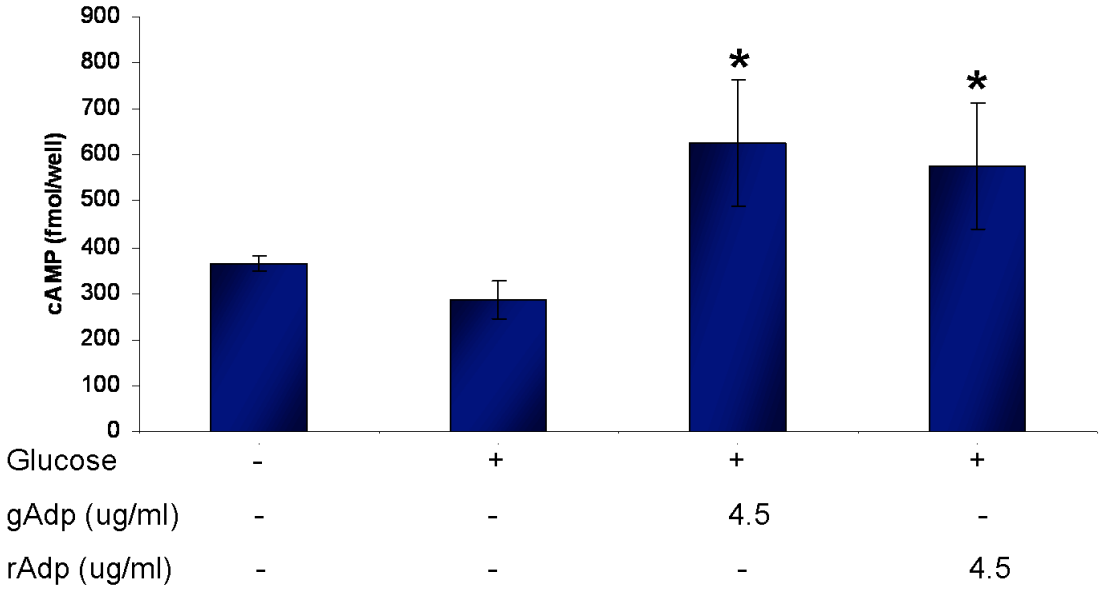


Figure 3.6. Effect of the released gAdp (rAdp) from the polymer gel on activating cAMP compared to the intact gAdp. This confirms the preservation of gAdp activity. The data represents means ± SD of n=3. *P<0.05 vs. high glucose. Statistical analysis was corrected after publication. A two-way ANOVA was performed with Bonferroni's posttest.

The polymer gel was not added directly to the cells due to its viscosity. However, the protein release could be achieved in animal models. This was performed in our laboratory using other proteins. The characterization of the gAdp delivery system raises the possibility to be used therapeutically in animal models.

Conclusions

The gAdp effectively suppressed excess ROS-production in the *in vitro* BAECs under high-glucose condition via cAMP pathway. The controlled release profile of gAdp from the polymer gel was achieved showing almost 100% sustained release over 27 days with no initial burst release. The released gAdp preserved its activity in reducing ROS and activating cAMP. These data raise the possibility that injectable gels containing gAdp could be used therapeutically to reduce ROS in the blood vessels of animals with diabetes. This released adiponectin might compensate for the reduced adiponectin concentrations in the blood stream of diabetic or obese animals. This study provides a new direction for developing controlled release dosage form of gAdp as a therapeutic tool for oxidative stress related pathology in patients with diabetes. The results provide a framework for developing scientific foundation for further work involving *in vivo* studies.

Acknowledgments

This work was partially supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (F104AA010004-06A0101-00410, R01-2007-000-20602-0 (2007)) and Seoul R&BD program (CR070027) in Korea.

References

1. Nathan, D. M., Cleary, P. A., Backlund, J. Y., Genuth, S. M., Lachin, J. M., Orchard, T. J., Raskin, P., and Zinman, B. (2005) Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes, *N. Engl. J. Med. 353*, 2643-2653.

2. Kuroki, T., Isshiki, K., and King, G. L. (2003) Oxidative stress: the lead or supporting actor in the pathogenesis of diabetic complications, *J. Am. Soc. Nephrol. 14*, S216-220.

3. Brownlee, M. (2005) The pathobiology of diabetic complications: a unifying mechanism, *Diabetes 54*, 1615-1625.

4. Ohara, Y., Peterson, T. E., and Harrison, D. G. (1993) Hypercholesterolemia increases endothelial superoxide anion production, *J. Clin. Invest. 91*, 2546-2551.

5. Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, B. A., Griendling, K. K., and Harrison, D. G. (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone, *J. Clin. Invest. 97*, 1916-1923.

6. Hattori, Y., Kawasaki, H., Abe, K., and Kanno, M. (1991) Superoxide dismutase recovers altered endothelium-dependent relaxation in diabetic rat aorta, *Am. J. Physiol. 261*, H1086-1094.

7. van der Loo, B., Labugger, R., Skepper, J. N., Bachschmid, M., Kilo, J., Powell, J. M., Palacios-Callender, M., Erusalimsky, J. D., Quaschning, T., Malinski, T., Gygi, D., Ullrich, V., and Luscher, T. F. (2000) Enhanced peroxynitrite formation is associated with vascular aging, *J. Exp. Med. 192*, 1731-1744.

8. Laurindo, F. R., de Souza, H. P., Pedro Mde, A., and Janiszewski, M. (2002) Redox aspects of vascular response to injury, *Methods Enzymol. 352*, 432-454.

9. Park, J. H., Lee, M., and Kim, S. W. (2006) Non-viral adiponectin gene therapy into obese type 2 diabetic mice ameliorates insulin resistance, *J. Control. Release 114*, 118-125.

10. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuzawa, Y. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity, *Biochem. Biophys. Res. Commun. 257*, 79-83.

11. Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., and Tataranni, P. A. (2001) Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia, *J. Clin. Endocrinol. Metab. 86*, 1930-1935.

12. Motoshima, H., Wu, X., Mahadev, K., and Goldstein, B. J. (2004) Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL, *Biochem. Biophys. Res. Commun. 315*, 264-271.

13. Costacou, T., Zgibor, J. C., Evans, R. W., Otvos, J., Lopes-Virella, M. F., Tracy, R. P., and Orchard, T. J. (2005) The prospective association between adiponectin and coronary artery disease among individuals with type 1 diabetes. The Pittsburgh Epidemiology of Diabetes Complications Study, *Diabetologia 48*, 41-48.

14. Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., and Shimomura, I. (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome, *J. Clin. Invest. 114*, 1752-1761.

15. Nakanishi, S., Yamane, K., Kamei, N., Nojima, H., Okubo, M., and Kohno, N. (2005) A protective effect of adiponectin against oxidative stress in Japanese Americans: the association between adiponectin or leptin and urinary isoprostane, *Metabolism 54*, 194-199.

16. Ouedraogo, R., Wu, X., Xu, S. Q., Fuchsel, L., Motoshima, H., Mahadev, K., Hough, K., Scalia, R., and Goldstein, B. J. (2006) Adiponectin suppression of high-glucose-induced reactive oxygen species in vascular endothelial cells: evidence for involvement of a cAMP signaling pathway, *Diabetes 55*, 1840-1846.

17. Kissel, T., Li, Y., Gorich, S., Volland, C., and Koneberg, R. (1996) Parenteral protein delivery systems using biodegradable ABA-block-copolymers, *J. Control. Release 39*, 315-326.

18. Duvvuri, S., Janoria, K. G., and Mitra, A. K. (2005) Development of a novel formulation containing poly(d,l-lactide-co-glycolide) microspheres dispersed in PLGA-PEG-PLGA gel for sustained delivery of ganciclovir, *J. Control. Release 108*, 282-293.

19. Chang, C. W., Choi, D., Kim, W. J., Yockman, J. W., Christensen, L. V., Kim, Y. H., and Kim, S. W. (2007) Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle, *J. Control. Release 118*, 245-253.

20. Zentner, G. M., Rathi, R., Shih, C., McRea, J. C., Seo, M. H., Oh, H., Rhee, B. G., Mestecky, J., Moldoveanu, Z., Morgan, M., and Weitman, S. (2001) Biodegradable block copolymers for delivery of proteins and water-insoluble drugs, *J. Control. Release 72*, 203-215.

21. Kissel, T., Li, Y., and Unger, F. (2002) ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins, *Adv. Drug Deliv. Rev. 54*, 99-134.

22. Chen, S., Pieper, R., Webster, D. C., and Singh, J. (2005) Triblock copolymers: synthesis, characterization, and delivery of a model protein, *Int. J. Pharm. 288*, 207-218.

23. Laurent, A., Nicco, C., Chereau, C., Goulvestre, C., Alexandre, J., Alves, A., Levy, E., Goldwasser, F., Panis, Y., Soubrane, O., Weill, B., and Batteux, F. (2005) Controlling tumor growth by modulating endogenous production of reactive oxygen species, *Cancer Res. 65*, 948-956.

24. Wu, X., Mahadev, K., Fuchsel, L., Ouedraogo, R., Xu, S. Q., and Goldstein, B. J. (2007) Adiponectin Suppresses IkB Kinase Activation Induced by Tumor Necrosis Factor-{alpha} or High Glucose in Endothelial Cells: Role of cAMP and AMP Kinase Signaling, *Am. J. Physiol. Endocrinol. Metab*. *293*, E1836-E1844.

25. Wong, E. T., Alsop, D., Lee, D., Tam, A., Barron, L., Bloom, J., Gautam, S., and Wu, J. K. (2008) Cerebrospinal fluid matrix metalloproteinase-9 increases during treatment of recurrent malignant gliomas, *Cerebrospinal Fluid Res. 5*, 1.

26. Aronson, D. (2008) Hyperglycemia and the pathobiology of diabetic complications, *Adv Cardiol 45*, 1-16.

CHAPTER 4

SOLID PHASE SYNTHESIS OF MITOCHONDRIAL TRIPHENYLPHOSPHONIUM-VITAMIN E USING A LYSINE LINKER FOR REVERSAL OF OXIDATIVE STRESS

Abstract

Mitochondrial targeting of antioxidants has been an area of interest due to the mitochondria's role in producing and metabolizing reactive oxygen species. Antioxidants have been conjugated to a lipophilic cation to increase their mitochondrial targeting. This paper describes the synthesis of mitochondria targeted vitamin E. We report a faster and a more efficient method to conjugate vitamin E to triphenylphosphonium cation via a lysine linker using solid phase synthesis. The efficacy of the final product was tested in bovine aortic endothelial cells and type 2 diabetic db/db mice to examine reduction in oxidative stress and mitochondrial targeting.

This chapter has been submitted to the *Journal of Organic Chemistry*.
Mossalam M., Soto J., Lim C.S., Abel E.D.
Mitochondrial isolation was performed by J.S. The rest of the experiments were performed by M.M.

Introduction

Metabolic syndrome, a group of risk factors that increase the risk of cardiovascular disease and diabetes, has become increasingly common in the United States (*1*). Some of the metabolic risk factors associated with metabolic syndrome are abdominal obesity, elevated blood pressure, hyperglycemia, insulin resistance, and atherogenic dyslipidemia (*2, 3*). These factors are associated with elevated rates of cardiovascular events such as sudden death, acute myocardial infarction, and cerebrovascular accidents. Reducing the risk of cardiovascular disease and type 2 diabetes are the primary goals of clinical management of the metabolic syndrome. Diabetic patients have a higher risk of dying of a heart attack or a stroke than non-diabetic patients (*4*). Endothelial dysfunction in diabetic patients is mainly caused by hyperglycemia, which increases ROS production (*5-7*). Increased production of systemic and vascular ROS leads to reduced endothelial nitric oxide bioactivity, increased expression of cell surface adhesion molecules, and inflammatory changes contributing to vascular, cellular, and tissue damage (*8-12*). Various studies have suggested that the complex process of endothelial ROS generation may be derived from several cellular sources, including NADPH-dependent oxidases, complex III of the electron transport chain in mitochondria, and multiple signaling cascades (*7, 13-16*).

The mitochondria play roles in cellular energy metabolism, apoptosis, Ca^{2+} homeostasis, and cell signaling (*17-20*). Because of these roles, the mitochondria have been under investigation for therapeutic targeting. Myocardial mitochondria are involved in the generation of energy, the regulation of apoptosis, and the generation and detoxification of ROS (*21-23*). Disrupting the mitochondrial Ca^{2+}, ATP, or ROS

metabolism plays a role in different diseases such as diabetes, obesity, heart failure, stroke, aging, cancer, and neurodegenerative diseases (*24*). The mitochondria utilize 90% of consumed oxygen for ATP synthesis and oxidative phosphorylation (*25*). This process in the electron transfer chain is responsible for ROS production. The cell has its own antioxidant defenses such as glutathione, catalase, and superoxide dismutase to prevent oxidative stress (*26-30*). Maintaining ROS/antioxidant ratio is imperative for cell signaling (*25*). Any imbalance between ROS generation and destruction is associated with chronic disease (*31*). Since the mitochondria produces and metabolizes ROS, targeting antioxidants to the mitochondria has been a focus of interest. This is achieved by conjugating an antioxidant to a lipophilic cation. The positive charge enables mitochondrial accumulation 100-1000 times higher due to the high inner mitochondrial membrane potential (*32-34*).

Conjugating an antioxidant such as vitamin E to a lipophilic cation can be a promising approach for reversing oxidative damage. Vitamin E is a fat-soluble antioxidant with eight naturally occurring vitamer forms. The most common forms are γ- and α-tocopherol. Vitamin E has a positive effect on insulin sensitivity and the prevention of type-2 diabetes (*35-37*) due to its antioxidant capacity (*38, 39*). Alpha-tocopherol is a chain-breaking antioxidant, where it interrupts the formation of lipid-derived oxygen- and carbon-centered free radicals (*25*). These radicals are formed via a chain reaction: initiation, propagation, and termination. Clinical trials with antioxidants have failed to show any significant clinical benefit. Meanwhile, administering high doses of vitamin E may be harmful (*40*). There is a possibility that this failure is due to the lack of antioxidant accumulation in the mitochondria, the proper target for scavenging ROS (*25*).

Targeting vitamin E conjugated to triphenylphosphonium (TPP^+) to the mitochondria has a better effect in reducing ROS than vitamin E by itself (*41, 42*).

The aim of this study was to design a faster and a more efficient method to conjugate vitamin E to TPP^+ (MitoE) using a lysine linker and solid phase synthesis. We also tested the efficacy of the new conjugated MitoE in decreasing ROS in bovine aortic endothelial cells (BAEC) and in targeting the mitochondria in type 2 diabetic db/db mice (*43*).

Materials and Methods

Solid Phase Synthesis of MitoE

Resin Fmoc Deprotection

The fluorenylmethoxycarbonyl (Fmoc) group was deprotected on the 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin (Rink Amide MBHA resin, EMD Chemicals, Germany). The resin (25 mg) was dissolved in 6 ml dimethylformamide (DMF), loaded into a fritted column (Grace Davison Discovery Sciences, Deerfield, IL) and washed twice in DMF. It was then incubated with 20% piperidine (Sigma-Aldrich, St. Louis, MO) in DMF for 15 minutes at room temperature. After the deprotection was complete, the reaction column was drained and washed with DMF.

Lysine Coupling

Three equivalents of Fmoc-N-ε-4-methyltrityl-L-lysine (Fmoc-Lys[Mtt]-OH, Anaspec, Fremont, CA) was combined with 5 equivalents of 2-(1H-Benzotriazole-1-yl)-

1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, EMD Chemicals), 5 equivalents of HOBt-6-carboxamidomethyl polystyrene (HOBt, EMD Chemicals), and 5 equivalents of *N*,*N*-Diisopropylethylamine (DIPEA, Sigma Aldrich) in DMF. This coupling solution was then added to the resin. The mixture was agitated for 5 hours at room temperature.

Lysine Fmoc De-protection and TPP Coupling

The Fmoc on the lysine was deprotected as mentioned above. Subsequently, 5 equivalents of (3-carboxypropyl)TPP (Sigma Aldrich) was coupled similarly to the Fmoc-Lys(Mtt)-OH coupling mentioned above.

Lysine Mtt Deprotecting and Vitamin E Coupling

The Mtt group on the lysine was de-protected by incubation with 94% dichloromethane (DCM, Sigma Aldrich), 5% Triisopropylsilane (Tis, Sigma Aldrich), and 1% Trifluoroacetic acid (TFA, Sigma Aldrich) for 15 minutes. The column was then drained and washed with DMF. Later, 3 equivalents of 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-propanoic acid (α-CEHC, Cayman Chemical, Ann Arbor, MI) was coupled as described above.

Resin Cleavage

The resin was treated with 95% TFA, 2.5% water, and 2.5% Tis and agitated for 2 hours. The treatment allowed the cleavage of the final product from the resin. The filtrate was then collected.

Mass Spectroscopy

A sample of the filtrate was air-dried and then re-dissolved in methanol (Sigma Aldrich). The MALDI-TOF mass spectroscopy was performed at the University of Utah Core Facility. Full scan spectra were recorded by scanning a m/z range of 500–2000.

Animal Studies and Mitochondria Isolation

Two hundred μM of the MitoE (**8**) were given to type 2 diabetic db/db mice (n=4) in their drinking water for 2 weeks.(*44-46*) Another population (n=4) was given plain water (no MitoE) as a negative control. The hearts were minced in STE1 buffer (250 mmol/l sucrose, 5 mmol/l Tris/HCL, 2mmol/l EGTA, pH 7.4) and then incubated in STE2 buffer (STE1 containing [wt/vol] 0.5% BSA, 5mmol/l $MgCl_2$, 1 mmol/l ATP, and 2.5 units/ml protease type VIII from Bacillus licheniformis) for 4 minutes on ice to digest.(*44*) The mixture was then diluted in STE1 buffer and homogenized using a Teflon pistil in a Potter- Elvejhem glass homogenizer. The homogenate was centrifuged at 8000g for 10 minutes at 4°C. Subsequently, the pellet was resuspended in STE1 buffer and centrifuged at 700g for 10 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged at 8000g for 10 minutes at 4°C. The final mitochondrial pellet was diluted in STE1 buffer to a final concentration of 0.1 mg/ml. All animal experiments were conducted and cared for under an IACUC approved protocol from the University of Utah.

Liquid Chromatograph/Mass Spectrometry (LC/MS)

The mitochondrial fraction was sonicated (Branson, Danbury, CT) for 5 seconds with maximum speed in an ice bath then stirred for 30 seconds. The sonication and stirring were repeated six times. The MitoE standard curve was plotted by six-point dilutions: 0, 0.5, 1, 2, 4, and 8 μg/ml (*47*). Samples (mitochondrial fraction and plasma) and standard controls were then analyzed on the LC/MS, University of Utah Department of Chemistry. The analysis was controlled by MassLynx mass spectrometry software.

Cell Culture and ROS Measurement

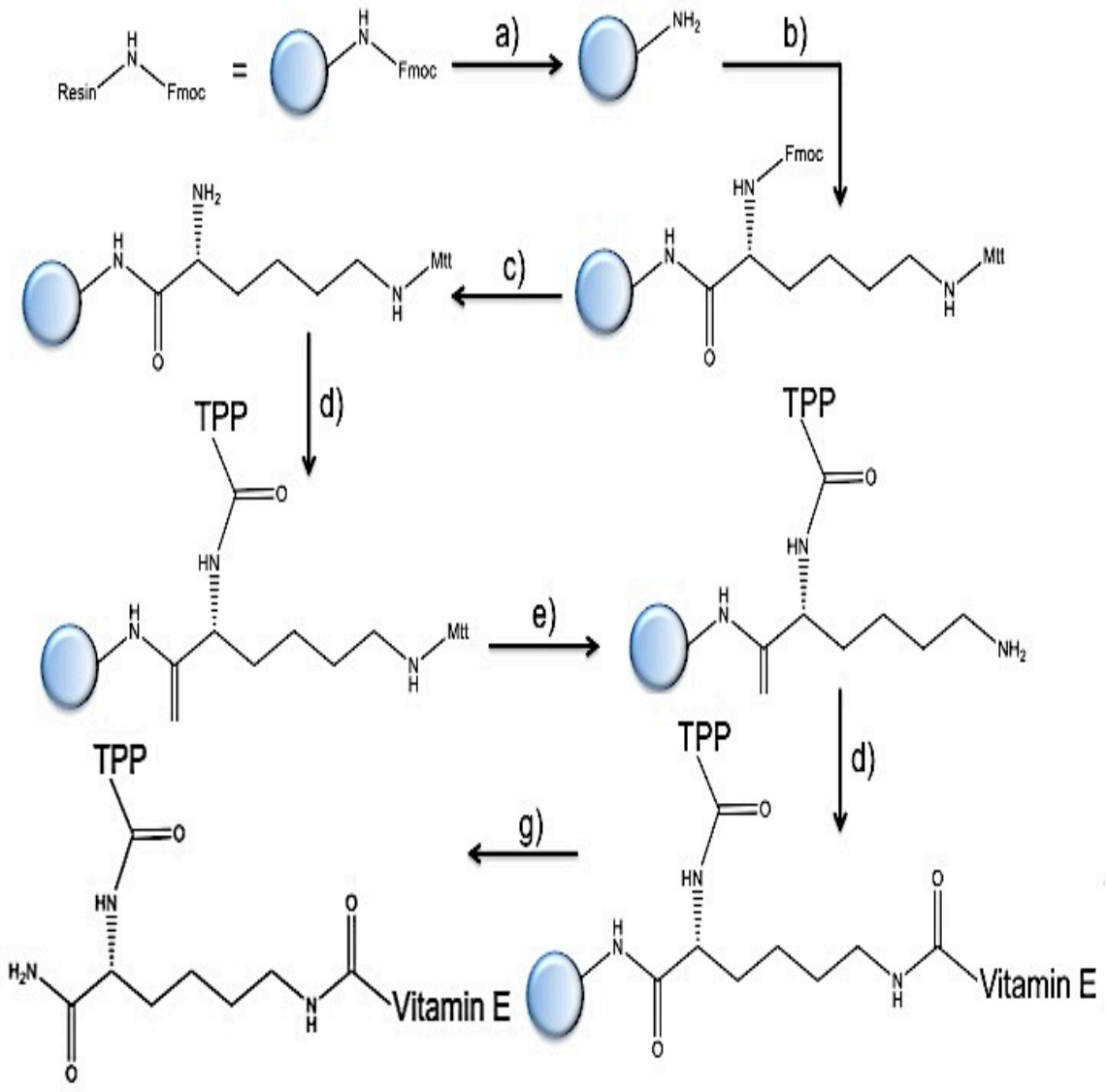
BAECs were grown as monolayers in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen). The cells were incubated in a 5% CO_2 incubator at 37ºC. A density of 2×10^5 cells were seeded in 6-well plates and treated with 5 mM or 25mM glucose (*23*). The cells treated with 25 mM glucose were then incubated with 2 μM Vitamin E (Sigma Aldrich) or 2 μM of our MitoE product for 36 hours. The cells were then treated with 10 μM 5-(and-6)-chlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen) for 30 minutes. Cells were then collected and suspended in PBS. Subsequently, they were analyzed using flow cytometry at the University of Utah Core Facility (*48*). The experiment was repeated in triplicate (n=3). The data were presented as the mean ± standard error. Statistical analysis was performed by using one-way ANOVA with Tukey's posttest. A value $p<0.05$ was considered significant.

Results and Discussion

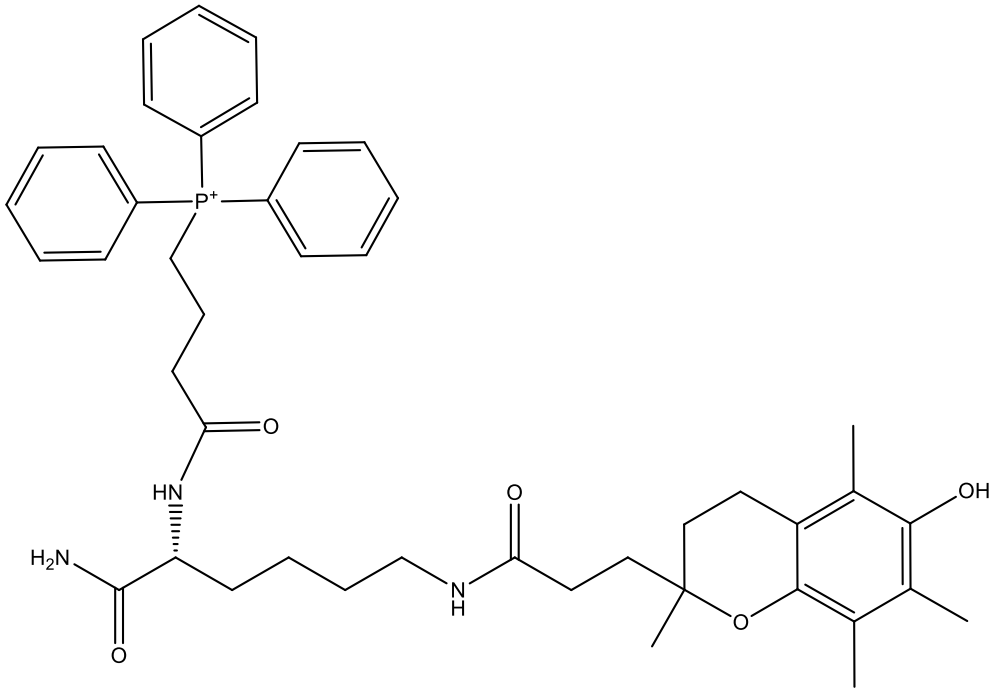
We envisaged a method to conjugate vitamin E to TPP. Such conjugation has been previously described. The literature precedent for this developed method was conducted with a more sophisticated scheme (*49*). However our goal was to design a more faster and efficient method using a lysine linker and solid phase synthesis. This method does not require isolation of synthetic intermediates while reagents and by-products are washed away after each step.

We used a lysine linker with two protecting groups (Fmoc and Mtt), which enabled the conjugation of TPP and vitamin E (Scheme 4.1). The masked lysine was coupled onto the Rink Amide MBHA resin. HBTU and HOBt were used to enhance the coupling rate (*50, 51*). The Fmoc was then deprotected to allow for (3-carboxypropyl)TPP conjugation through its carboxylic acid group forming an amide bond. The Mtt protecting group was then removed. The removal of the protecting group enabled the carboxylic acid on α-CEHC to form an amide bond with the lysine linker. The final product, TPP-Lysine-Vitamin E (MitoE), was then released from the resin via treatment with 95% TFA.

The final product (Figure 4.1) was characterized by MALDI-TOF mass spectrometry (Figure 4.2). The molecular weight peak was at 736.39, which corresponds to the expected peak for the MitoE generate by ChemDraw software. The mass spectrometry data also shows virtually no traces of by-products, reagents or synthetic intermediates.



Scheme 4.1. Solid phase synthesis of MitoE *Reagents and conditions:* a) 20% piperidine, DMF. b) Fmoc-Lys[Mtt]-OH, HBTU, HOBt, DIPEA, DMF. c) 20% piperidine, DMF. d) (3-carboxyproppyl)TPP, HBTU, HOBt, DIPEA, DMF. e) 94% DCM, 5% Tis, 1% TFA. f) α-CEHC, HBTU, HOBt, DIPEA, DMF. g) 95% TFA, 2.5% water, 2.5% Tis.



(4-(((2*R*)-1-amino-6-(3-(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)propanamido)-1-oxohexan-2-yl)amino)-4-oxobutyl)triphenylphosphonium

Chemical Formula: $C_{44}H_{55}N_3O_5P^+$

Exact Mass: 736.39

Molecular Weight: 736.90

m/z: 736.39 (100.0%), 737.39 (48.4%), 738.39 (12.6%), 739.40 (2.3%), 737.38 (1.1%)

Elemental Analysis: C, 71.72; H, 7.52; N, 5.70; O, 10.86; P, 4.20

Figure 4.1. The structure, nomenclature, and chemical formula of MitoE synthesized. Using ChemDraw Ultra software, it also shows the expected molecular weight and mass spectrometry analysis.

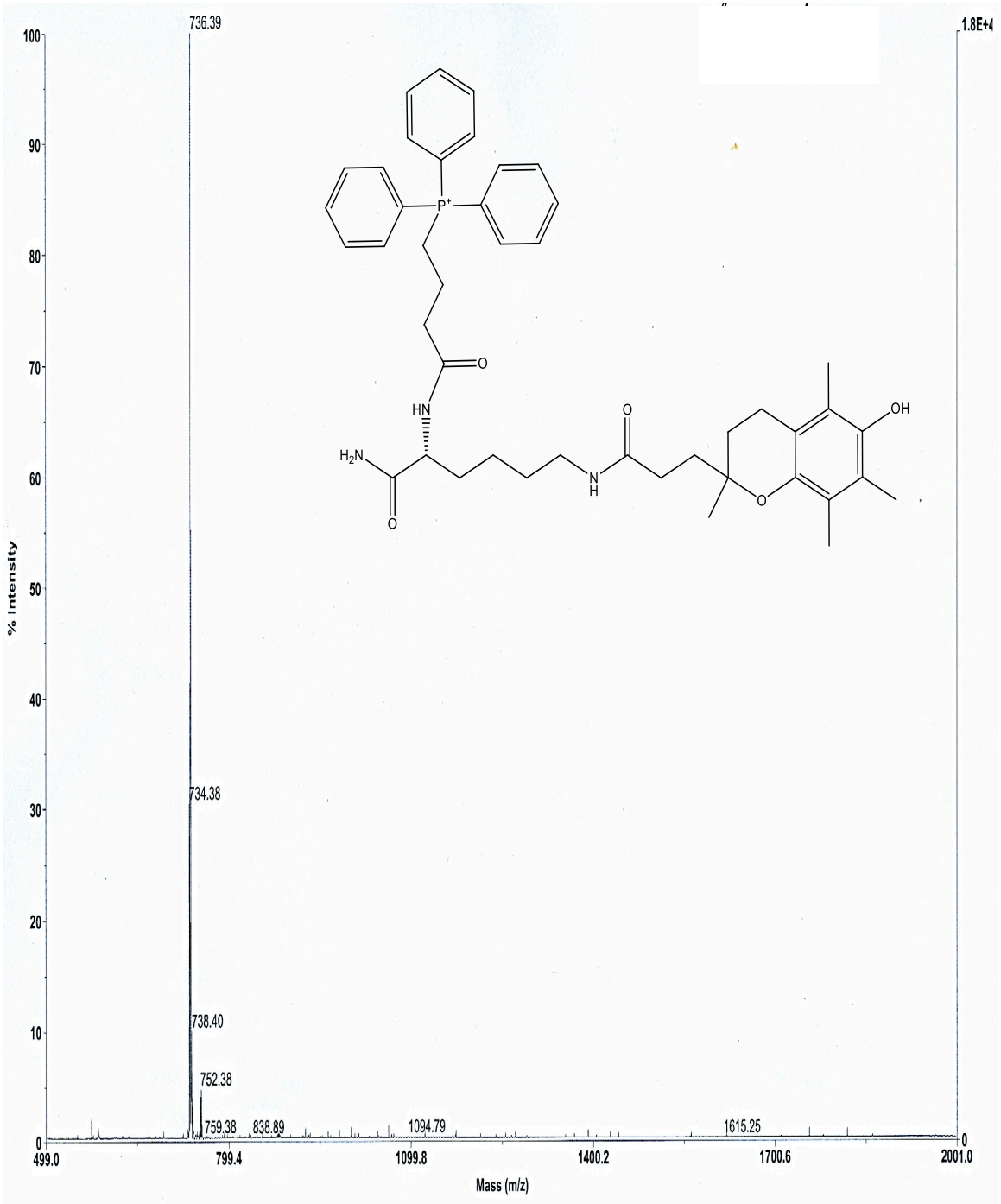


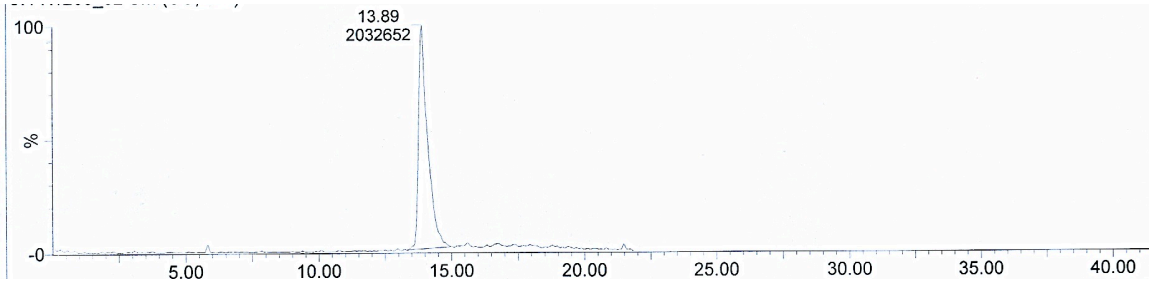
Figure 4.2. The MALDI-TOF mass spectrometry of sample from resin cleavage (MitoE). The 736.39 m/z is the actual mass.

In an effort to investigate if the TPP conjugation to vitamin E via a lysine linker would increase mitochondrial targeting, an *in vivo* experiment was performed. Highly insulin resistant db/db mice were provided with 2 μM of the MitoE in their dinking water for two weeks. Their plasma was collected and hearts were harvested to isolate myocardium mitochondria. The isolated mitochondria were then lysed. The concentrations of MitoE in the collected samples were simultaneously measured against a concentration standard curve. The retention times for the MitoE standard and samples are shown as 13.9 and 13.6 minutes respectively (Figure 4.3). The MitoE amount in the isolated mitochondria was 0.775 ± 0.137 μg/0.1g of mitochondria while the plasma concentration was 1.78 ± 0.305 μg/ml. The untreated mice showed no traces of MitoE (**8**) in the isolated mitochondria or plasma.

To further explore the antioxidant activity of the conjugated MitoE, BAECs were studied for ROS measurements. The endothelial cells were incubated with low (5 mM) and high (25 mM) glucose concentrations. The cells incubated under hyperglycemic conditions showed an increase in ROS production. Flow cytometry data also showed reduction in ROS production in the hyperglycemic cells treated with MitoE (Figure 4.4). Vitamin E conjugated to TPP via a lysine linker (MitoE) showed better effect than vitamin E alone.

In summary, the conjugation of Vitamin E to TPP was achieved in a fast and more efficient way using a lysine linker and solid phase synthesis. The conjugated product was effective in reducing oxidative stress in BAECs and targeting the mitochondria in type 2 diabetic db/db mice. This chemistry provides the framework for further products to be

A.



B.

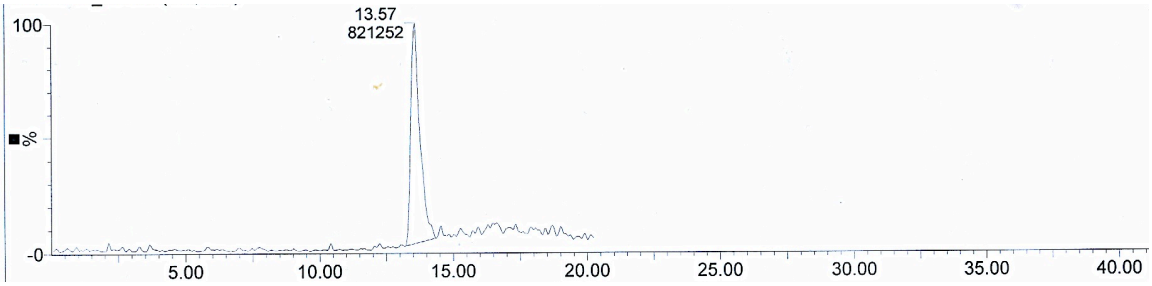


Figure 4.3. LC/MS data showing the retention time of (A) sample from resin cleavage, MitoE and (B) mitochondrial lysate of MitoE treated mice.

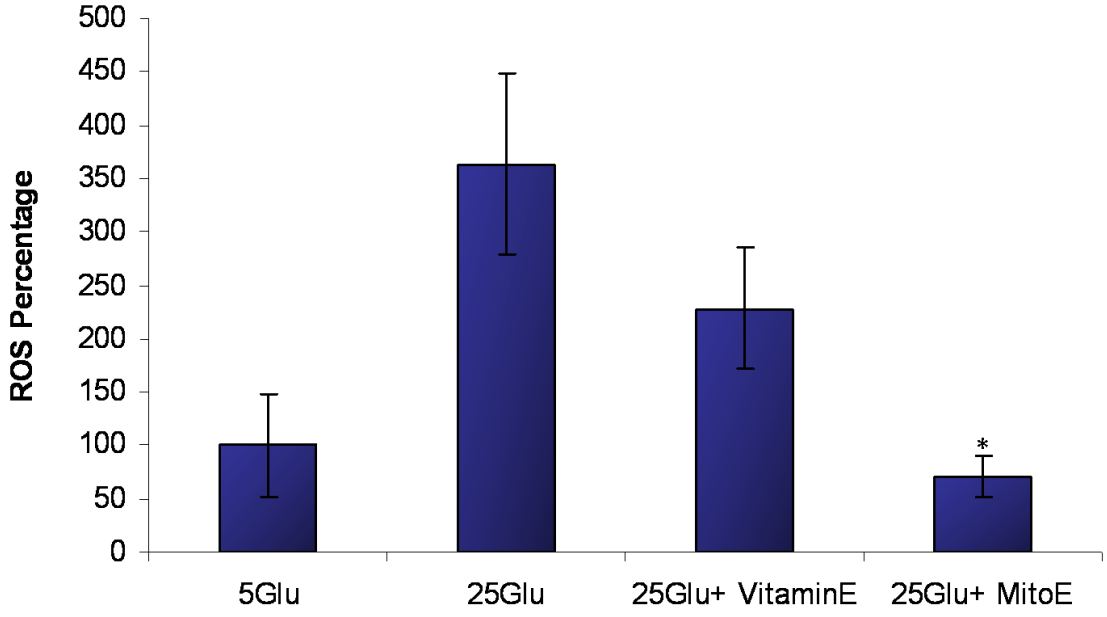


Figure 4.4. Effect of Vitamin E and MitoE on reducing ROS induced by high glucose in endothelial cells. ROS was measured via FACSCAN. Data are expressed as the percent of basal (5 mM glucose). Statistical analysis was performed by using one-way ANOVA with Tukey's posttest. * $p<0.05$ compared to 25 mM glucose treated cells.

explored. TPP conjugation using this method should be investigated with other antioxidants such as co-enzyme Q and quercetin. Different amino acids could also be used as linkers to investigate the effect of their length (such as lysine versus glycine).

References

1. Ford, E. S., Giles, W. H., and Dietz, W. H. (2002) Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey, *JAMA 287*, 356-359.

2. Santos, A. C., and Barros, H. (2007) Impact of metabolic syndrome definitions on prevalence estimates: a study in a Portuguese community, *Diab. Vasc. Dis. Res. 4*, 320-327.

3. Coelho, F. A., Moutinho, M. A., de Miranda, V. A., Tavares, L. R., Rachid, M., Rosa, M. L., and Mesquita, E. T. (2007) The association between metabolic syndrome and its components and heart failure in patients referred to a primary care facility, *Arq. Bras. Cardiol. 89*, 42-51.

4. Haffner, S., and Taegtmeyer, H. (2003) Epidemic obesity and the metabolic syndrome, *Circulation 108*, 1541-1545.

5. Nathan, D. M., Cleary, P. A., Backlund, J. Y., Genuth, S. M., Lachin, J. M., Orchard, T. J., Raskin, P., and Zinman, B. (2005) Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes, *N. Engl. J. Med. 353*, 2643-2653.

6. Kuroki, T., Isshiki, K., and King, G. L. (2003) Oxidative stress: the lead or supporting actor in the pathogenesis of diabetic complications, *J. Am. Soc. Nephrol. 14*, S216-220.

7. Brownlee, M. (2005) The pathobiology of diabetic complications: a unifying mechanism, *Diabetes 54*, 1615-1625.

8. Hink, U., Li, H., Mollnau, H., Oelze, M., Matheis, E., Hartmann, M., Skatchkov, M., Thaiss, F., Stahl, R. A., Warnholtz, A., Meinertz, T., Griendling, K., Harrison, D. G., Forstermann, U., and Munzel, T. (2001) Mechanisms underlying endothelial dysfunction in diabetes mellitus, *Circ. Res. 88*, E14-22.

9. Stone, J. R., and Collins, T. (2002) The role of hydrogen peroxide in endothelial proliferative responses, *Endothelium 9*, 231-238.

10. Taniyama, Y., and Griendling, K. K. (2003) Reactive oxygen species in the vasculature: molecular and cellular mechanisms, *Hypertension 42*, 1075-1081.

11. Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2003) Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction?, *Diabetes 52*, 1-8.

12. Ceriello, A., and Motz, E. (2004) Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited, *Arterioscler. Thromb. Vasc. Biol. 24*, 816-823.

13. Inoguchi, T., Sonta, T., Tsubouchi, H., Etoh, T., Kakimoto, M., Sonoda, N., Sato, N., Sekiguchi, N., Kobayashi, K., Sumimoto, H., Utsumi, H., and Nawata, H. (2003) Protein kinase C-dependent increase in reactive oxygen species (ROS) production in vascular tissues of diabetes: role of vascular NAD(P)H oxidase, *J. Am. Soc. Nephrol. 14*, S227-232.

14. Goldstein, B. J., and Scalia, R. (2004) Adiponectin: A novel adipokine linking adipocytes and vascular function, *J. Clin. Endocrinol. Metab. 89*, 2563-2568.

15. Kimura, S., Zhang, G. X., Nishiyama, A., Shokoji, T., Yao, L., Fan, Y. Y., Rahman, M., and Abe, Y. (2005) Mitochondria-derived reactive oxygen species and vascular MAP kinases: comparison of angiotensin II and diazoxide, *Hypertension 45*, 438-444.

16. Andresen, J. J., Faraci, F. M., and Heistad, D. D. (2004) Vasomotor responses in MnSOD-deficient mice, *Am. J. Physiol. Heart Circ. Physiol. 287*, H1141-1148.

17. Papa, S., and Skulachev, V. P. (1997) Reactive oxygen species, mitochondria, apoptosis and aging, *Mol. Cell Biochem. 174*, 305-319.

18. Green, D. R., and Reed, J. C. (1998) Mitochondria and apoptosis, *Science 281*, 1309-1312.

19. Pinkoski, M. J., Waterhouse, N. J., and Green, D. R. (2006) Mitochondria, apoptosis and autoimmunity, *Curr Dir Autoimmun. 9*, 55-73.

20. Horobin, R. W., Trapp, S., and Weissig, V. (2007) Mitochondriotropics: a review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria, *J. Control. Release 121*, 125-136.

21. Brennan, J. P., Southworth, R., Medina, R. A., Davidson, S. M., Duchen, M. R., and Shattock, M. J. (2006) Mitochondrial uncoupling, with low concentration FCCP, induces ROS-dependent cardioprotection independent of KATP channel activation, *Cardiovasc. Res. 72*, 313-321.

22. Tsutsui, H., Kinugawa, S., and Matsushima, S. (2011) Oxidative Stress and Heart Failure, *Am. J. Physiol. 301*, H2181-2190.

23. Mossalam, M., Jeong, J. H., Abel, E. D., Kim, S. W., and Kim, Y. H. (2008) Reversal of oxidative stress in endothelial cells by controlled release of adiponectin, *J. Control. Release 130*, 234-237.

24. Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., and Sheu, S. S. (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle, *Am. J. Physiol. 287*, C817-833.

25. Sheu, S. S., Nauduri, D., and Anders, M. W. (2006) Targeting antioxidants to mitochondria: a new therapeutic direction, *Biochimica. et Biophysica. Acta. 1762*, 256-265.

26. Abid, M. R., Schoots, I. G., Spokes, K. C., Wu, S. Q., Mawhinney, C., and Aird, W. C. (2004) Vascular endothelial growth factor-mediated induction of manganese superoxide dismutase occurs through redox-dependent regulation of forkhead and IkappaB/NF-kappaB, *J. Biol. Chem. 279*, 44030-44038.

27. Zhang, H. J., Drake, V. J., Xu, L., Hu, J., Domann, F. E., Oberley, L. W., and Kregel, K. C. (2002) Redox regulation of adenovirus-induced AP-1 activation by overexpression of manganese-containing superoxide dismutase, *J. Virol. 76*, 355-363.

28. Li, S., Yan, T., Yang, J. Q., Oberley, T. D., and Oberley, L. W. (2000) The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase, *Cancer Res. 60*, 3927-3939.

29. Warner, B. B., Stuart, L., Gebb, S., and Wispe, J. R. (1996) Redox regulation of manganese superoxide dismutase, *Am. J. Physiol. 271*, L150-158.

30. Schiavone, J. R., and Hassan, H. M. (1988) The role of redox in the regulation of manganese-containing superoxide dismutase biosynthesis in Escherichia coli, *J. Biol. Chem. 263*, 4269-4273.

31. Ross, E. A., Koo, L. C., and Moberly, J. B. (1997) Low whole blood and erythrocyte levels of glutathione in hemodialysis and peritoneal dialysis patients, *Am. J. Kid. Dis. 30*, 489-494.

32. Ross, M. F., Kelso, G. F., Blaikie, F. H., James, A. M., Cocheme, H. M., Filipovska, A., Da Ros, T., Hurd, T. R., Smith, R. A., and Murphy, M. P. (2005) Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology, *Biochem. 70*, 222-230.

33. Adlam, V. J., Harrison, J. C., Porteous, C. M., James, A. M., Smith, R. A., Murphy, M. P., and Sammut, I. A. (2005) Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury, *FASEB J. 19*, 1088-1095.

34. Mossalam, M., Dixon, A. S., and Lim, C. S. (2010) Controlling subcellular delivery to optimize therapeutic effect, *Ther. Deliv. 1*, 169-193.

35. Paolisso, G., D'Amore, A., Giugliano, D., Ceriello, A., Varricchio, M., and D'Onofrio, F. (1993) Pharmacologic doses of vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetic patients, *Am. J. Clinical Nutrition 57*, 650-656.

36. Paolisso, G., Di Maro, G., Galzerano, D., Cacciapuoti, F., Varricchio, G., Varricchio, M., and D'Onofrio, F. (1994) Pharmacological doses of vitamin E and insulin action in elderly subjects, *Am. J. Clinical Nutrition 59*, 1291-1296.

37. Mayer-Davis, E. J., Costacou, T., King, I., Zaccaro, D. J., and Bell, R. A. (2002) Plasma and dietary vitamin E in relation to incidence of type 2 diabetes: The Insulin Resistance and Atherosclerosis Study (IRAS), *Diabetes Care 25*, 2172-2177.

38. Caballero, B. (1993) Vitamin E improves the action of insulin, *Nut. Rev. 51*, 339-340.

39. Vinayaga Moorthi, R., Bobby, Z., Selvaraj, N., and Sridhar, M. G. (2006) Vitamin E protects the insulin sensitivity and redox balance in rat L6 muscle cells exposed to oxidative stress, *Int. J. Clin. Chem. 367*, 132-136.

40. Miller, E. R., 3rd, Pastor-Barriuso, R., Dalal, D., Riemersma, R. A., Appel, L. J., and Guallar, E. (2005) Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality, *Ann. Int. Med. 142*, 37-46.

41. Dhanasekaran, A., Kotamraju, S., Kalivendi, S. V., Matsunaga, T., Shang, T., Keszler, A., Joseph, J., and Kalyanaraman, B. (2004) Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis, *J. Biol. Chem. 279*, 37575-37587.

42. Echtay, K. S., Murphy, M. P., Smith, R. A., Talbot, D. A., and Brand, M. D. (2002) Superoxide activates mitochondrial uncoupling protein 2 from the matrix side. Studies using targeted antioxidants, *J. Biol. Chem. 277*, 47129-47135.

43. Dray, C., Debard, C., Jager, J., Disse, E., Daviaud, D., Martin, P., Attane, C., Wanecq, E., Guigne, C., Bost, F., Tanti, J. F., Laville, M., Vidal, H., Valet, P., and Castan-Laurell, I. (2010) Apelin and APJ regulation in adipose tissue and skeletal muscle of type 2 diabetic mice and humans, *Am. J. Physiol. 298*, E1161-1169.

44. Bugger, H., Chen, D., Riehle, C., Soto, J., Theobald, H. A., Hu, X. X., Ganesan, B., Weimer, B. C., and Abel, E. D. (2009) Tissue-specific remodeling of the mitochondrial proteome in type 1 diabetic akita mice, *Diabetes 58*, 1986-1997.

45. Smith, R. A., Porteous, C. M., Gane, A. M., and Murphy, M. P. (2003) Delivery of bioactive molecules to mitochondria in vivo, *PNAS USA 100*, 5407-5412.

46. Buchanan, J., Mazumder, P. K., Hu, P., Chakrabarti, G., Roberts, M. W., Yun, U. J., Cooksey, R. C., Litwin, S. E., and Abel, E. D. (2005) Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity, *Endocrinol. 146*, 5341-5349.

47. Persson, X. M., Blachnio-Zabielska, A. U., and Jensen, M. D. (2010) Rapid measurement of plasma free fatty acid concentration and isotopic enrichment using LC/MS, *J. Lipid Res. 51*, 2761-2765.

48. Laurent, A., Nicco, C., Chereau, C., Goulvestre, C., Alexandre, J., Alves, A., Levy, E., Goldwasser, F., Panis, Y., Soubrane, O., Weill, B., and Batteux, F. (2005) Controlling tumor growth by modulating endogenous production of reactive oxygen species, *Cancer Res. 65*, 948-956.

49. Smith, R. A., Porteous, C. M., Coulter, C. V., and Murphy, M. P. (1999) Selective targeting of an antioxidant to mitochondria, *Eur. J. Biochem. 263*, 709-716.

50. Ambrosius, D., Casaretto, M., Gerardy-Schahn, R., Saunders, D., Brandenburg, D., and Zahn, H. (1989) Peptide analogues of the anaphylatoxin C3a; syntheses and properties, *J. Biol. Chem. Hoppe-Seyler 370*, 217-227.

51. Liley, M. J., Johnson, T., and Gibson, S. E. (2006) An improved aldehyde linker for the solid phase synthesis of hindered amides, *J. Org. Chem. 71*, 1322-1329.

CHAPTER 5

INDUCTION OF APOPTOSIS THROUGH COMPARTMENT SPECIFIC MITOCHONDRIAL TARGETING OF P53

Abstract

Targeting the tumor suppressor p53 to the mitochondria triggers a rapid apoptotic response as efficiently as transcription-dependent p53 (*1, 2*). p53 forms a complex with the anti-apoptotic Bcl-XL, which leads to Bak and Bax oligomerization resulting in apoptosis via mitochondrial outer membrane permeabilization (*3, 4*). Although p53 performs its main role in the mitochondrial outer membrane it also interacts with different proteins in the mitochondrial inner membrane and matrix (*5, 6*). To further investigate mitochondrial activity of p53, we targeted p53 to specific mitochondrial compartments. EGFP-p53 was fused to different mitochondrial targeting signals (MTSs) directing it to the mitochondrial outer membrane ("XL-MTS" from Bcl-XL; "TOM-MTS" from TOM20), the inner membrane ("CCO-MTS" from cytochrome c oxidase) or matrix ("OTC-MTS" from ornithine transcarbamylase). Fluorescence microscopy and a

---

This chapter has been submitted to *Molecular Pharmaceutics*.
Mossalam M., Matissek K.J., Okal A., Constance J.E., Lim C.S.
All work was performed by both M.M. and K.J.M. and are referred to as co-first authors. A.O performed part of the luciferase assay while J.E.C. helped with flow cytometry and image analysis.

p53 reporter dual luciferase assay demonstrated that fusing MTSs to p53 increased mitochondrial localization and nuclear exclusion depending on which MTS was used. To examine if the MTSs initiate mitochondrial damage, we fused each individual MTS to EGFP (a nontoxic protein) as negative controls. We performed caspase-9, TUNEL, Annexin-V, and 7-AAD apoptosis assays on T47D breast cancer cells transfected with mitochondrial constructs. Except for EGFP-XL, apoptotic potential was observed in all MTS-EGFP-p53 and MTS-EGFP constructs. In addition, EGFP-p53-XL showed the greatest significant increase in programmed cell death compared to its non-toxic MTS control (EGFP-XL). The apoptotic mechanism for each construct was further investigated using pifithrin-α (an inhibitor of p53 transcriptional activity), pifithrin-μ (a small molecule that reduces binding of p53 to Bcl-2 and Bcl-XL), and over-expressing the anti-apoptotic Bcl-XL. Unlike the MTSs from TOM, CCO, and OTC, which showed different apoptotic mechanisms, we conclude that p53 fused to the MTS from Bcl-XL performs its apoptotic potential exclusively through p53/Bcl-XL specific pathway.

Introduction

The tumor suppressor p53 stimulates a wide network of signals involved in DNA repair, cell cycle arrest, senescence and apoptosis (*7-11*). Although most of these effects can be linked to its role as a transcription factor, recent work has clearly demonstrated that p53 can cause apoptosis through its transcription-independent mitochondrial pathway (*3, 12*). A small but highly reproducible fraction of p53 translocates to the mitochondria at the onset of p53-dependent apoptosis (*12*). Translocation of p53 to the mitochondrial outer membrane triggers the release of cytochrome c and procaspase-3 activation. The

DNA binding domain of p53 (DBD, residues 239-248) forms inhibitory complexes with anti-apoptotic Bcl-XL and Bcl-2 proteins (Figure 5.1), which are located in the mitochondrial outer membrane (*13*). This induces oligomerization of Bak and Bax allowing them to form supramolecular pores (*4, 14, 15*). In addition, p53 activates, directly binds to, and induces oligomerization of Bak and Bax (*4, 15, 16*). The formation of permeability transition pores causes outer membrane rupture and releases cytochrome c from the intramembranous space into the cytosol triggering apoptosis via the apoptosome formation (Figure 5.1) (*17-19*). In addition, targeting p53 to the mitochondria triggers apoptosis faster than the transcription-dependent nuclear pathway (*1, 2*). In most cancer cells, p53 is not able to bind to Bcl-2 proteins due to missense mutations in the DBD of p53, demonstrating the importance of the DBD in mitochondrial apoptosis (*13*).

In addition to p53's role in the outer membrane, it also shows activity in the mitochondrial inner membrane and matrix. Under stress, p53 forms a complex with cyclophilin D, which is found in the mitochondrial inner membrane (*20*). This complex initiates the mitochondrial permeability transition pore, which leads to mitochondrial swelling and apoptosis (*21*). In the mitochondrial matrix, p53 interacts with manganese superoxide dismutase and reduces its activity. Due to this interaction, p53 interferes with the cellular oxidative defense mechanism resulting in reduced degradation of reactive oxygen species. This causes a decrease of mitochondrial potential and results in apoptosis (*5*).

Despite what is known, the mechanism of mitochondrial p53 activity is still under investigation. To elucidate this mechanism, we present data on delivering p53 to different

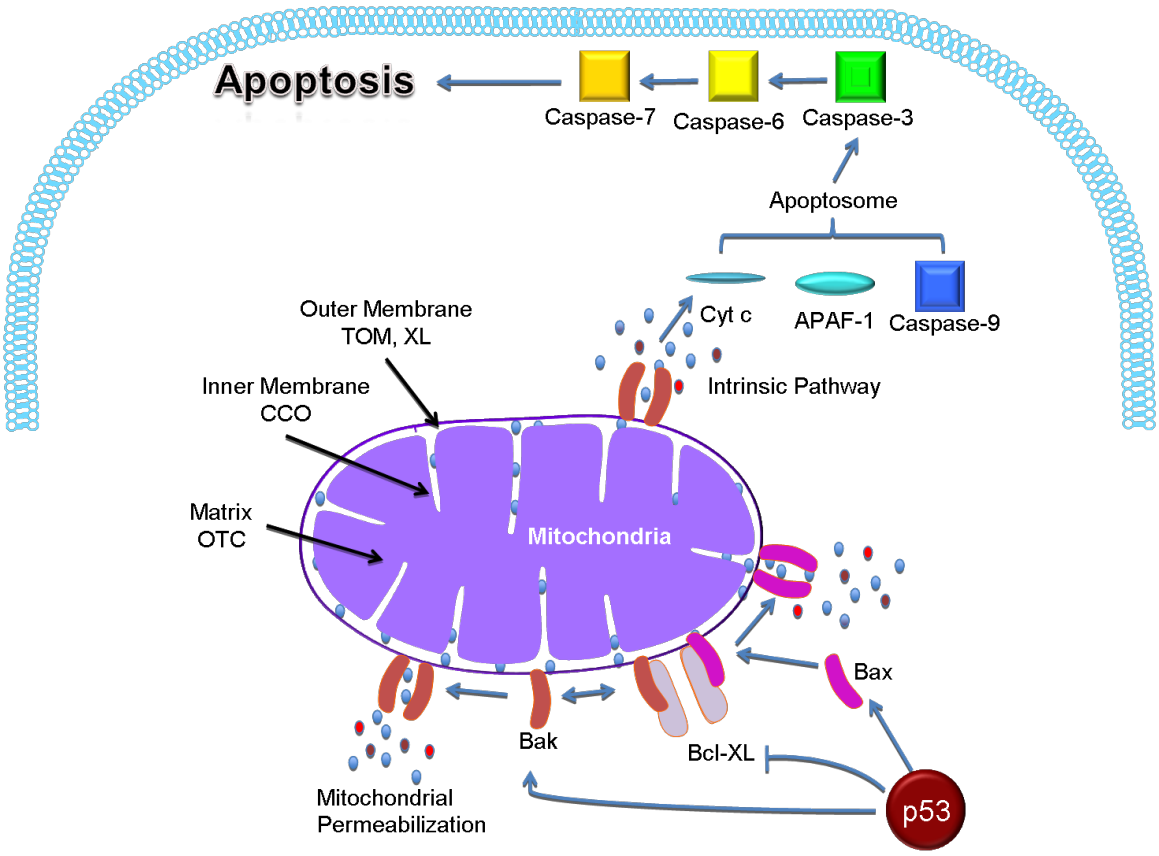


Figure 5.1. The mitochondrial apoptotic pathway of p53. When p53 is targeted to the mitochondria, it interacts with anti-apoptotic Bcl-XL, enables Bax and Bak oligomerization, and activates the intrinsic apoptotic pathway. The apoptosome (cytochrome c, APAF-1 and caspase-9) is triggered, leading to apoptosis via activation of caspases-3, -6, and -7. The left side of the diagram indicates the different subsections of the mitochondria targeted, including the outer membrane ("XL-MTS" from Bcl-XL; "TOM-MTS" from TOM20), the inner membrane ("CCO-MTS" from cytochrome c oxidase), and the matrix ("OTC-MTS" from ornithine transcarbamylase).

mitochondrial compartments. We fused p53 to different mitochondrial targeting signals (MTSs) targeting the mitochondrial outer membrane [MTS from Bcl-XL and MTS from the translocase of the outer membrane (TOM 20)], inner membrane (MTS from cytochrome c oxidase subunit VIII) and matrix (MTS from ornithine transcarbamylase) (Figure 5.1). These MTSs (Table 5.1) are thought to form α-helices, and are important for their recognition by translocation machineries in the mitochondrial outer (TOM complex) and inner (TIM complex) membranes (*22-25*). MTSs are mainly found on the amino terminus of mitochondrial proteins (*22*).

In this project, all MTSs used were cloned on the amino terminus except for XL, which is inherently found on the carboxy terminus (*26*). XL targets the outer surface of the mitochondria (*26-29*) while TOM is inserted into the outer membrane (*26*). CCO translocates the outer membrane and then becomes imbedded into the inner membrane (*30, 31*) while the OTC crosses both membranes and translocates to the matrix (*3, 32*). The purpose of this work was to determine if p53 targeted to different mitochondrial compartments exhibit differences in apoptotic potential.

Materials and Methods

EGFP-p53 plasmid (pEGFP-p53)

The DNA encoding p53 was amplified through PCR from pCMV-p53 wt (a generous gift from Dr. S. J. Baker, Addgene, Cambridge, MA) using the primers 5′-GCGCGCGCGCTCCGGAGCCATGGAGGAGCCGCAGT-3′ and 5′-GCGCGCGCGC-GGTACCTCAGTCTGAGTCAGGCCCTTCTGTC-3′. This was subcloned into the BspEI and KpnI restriction enzyme sites in pEGFP-C1 (Clontech, Mountain View, CA).

Table 5.1. A list of the mitochondrial targeting signals used in this paper. TOM and XL target the mitochondrial outer membrane, CCO translocates to the mitochondrial inner membrane, and the OTC localizes to the mitochondrial matrix.

Protein	Compartment	MTS Sequence
XL	Outer Membrane	RGPGIQKGPGEIQQMVPDRHDRGRRGAAGQPVQQKX
TOM	Outer Membrane	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPN
CCO	Inner Membrane	MSVLTPLLLRGLTGSARRLPVPRAKIHSL
OTC	Matrix	MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQ

pOTC-EGFP-p53 plasmid

An oligonucleotide encoding the mitochondrial targeting signal from OTC (including the Kozak region), 5′-CCGGTCGCCACCATGCTGTTTAATCTGAGG-ATCCTGTTAAACAATGCAGCTTTTAGAAATGGTCACAACTTCATGGTTCGAAA TTTTCGGTGTGGACAACCACTACAAAATAAAGTGCAGCGA-3′ was annealed to its complementary strand. This was then cloned at the amino terminus of EGFP-p53 at the AgeI site.

pTOM-EGFP-p53 and pCCO-EGFP-p53 plasmid

The OTC region from pOTC-EGFP-p53 was replaced with annealed oligonucleotides encoding the mitochondrial targeting signal from the TOM20 complex (TOM) at the AgeI site, 5′-GATCCCCGGTCGCCACCATGGTGGGTCGGAACAG-CGCCATCGCCGCCGGTGTATGCGGGGCCCTTTTCATTGGGTACTGCATCTACT TCGACCGCAAAAGACGAAGTGACCCCAACCGA-3′ and its reverse complement, or with oligonucleotides encoding the mitochondrial targeting signal from the cytochrome c oxidase (CCO) at the AgeI site, 5′-CCGGTCGCCACCATGTCCGTCCTGACGCCG-CTGCTGCTGCGGGGCTTGACAGGCTCGGCCCGGCGGCTCCCAGTGCCGCGCG CCAAGATCCATTCGTTGA-3′ and its reverse complement.

pEGFP-p53-XL plasmid

The mitochondrial signal from Bcl-XL was fused to the carboxy terminus of EGFP-p53 using the BamHI restriction site as follows. The XL oligonucleotide 5′-AGAAAGGGCCAGGAGAGATTCAACAGATGGTTCCTGACCGGCATGACCGTG

GCCGGCGTGGTGCTGCTGGGCAGCCTGTTCAGCAGAAAGTGA-3′ was annealed to its complimentary strand (with BamHI sticky ends). The p53 stop codon was then mutated (TGA to TTA) in pEGFP-p53-XL using the primers 5′-GAAGGGCCTGACT-CAGACTTAGGTACCGCGGGCCCGGGAT-3′ and the reverse complement.

pEGFP constructs with MTSs

Plasmids encoding OTC-EGFP, TOM-EGFP, CCO-EGFP, and EGFP-Bcl-XL were constructed using the same oligonucleotides and restriction sites mentioned above but inserted in pEGFP-C1 instead of pEGFP-p53.

MTS-EGFP-p53NLSmut

Mutations (K319T and K320T) in the nuclear localization signal (NLS) of p53 were introduced in all mitochondrial p53 constructs via QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) using the primers 5′-CTCTCCCCAGCC-AACGACGAAACCACTGG-3′ and its reverse complement.

Cell Lines and Transient Transfections

1471.1 murine adenocarcinoma cells (gift of G. Hager, NCI, NIH), MCF-7 human breast adenocarcinoma cells (ATCC, Manassas, VA), and T47D human ductal breast epithelial tumor cell line (ATCC) were grown as monolayers in DMEM (1471.1) or RPMI (MCF-7 and T47D) (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin-glutamine (Invitrogen), and 0.1% gentamicin (Invitrogen). In addition, T47D and MCF-7 media was supplemented with 4

mg/L insulin (Sigma, St. Louis, MO). The cells were maintained in a 5% CO_2 incubator at 37ºC. 7.5×10^4 cells for 1471.1 and 3.0×10^5 cells for MCF-7 and T47D were seeded in 6-well plates (Greiner Bio-One, Monroe, NC) or 2-well live cell chambers (Nalgene Nunc, Rochester, NY). Transfections were carried out 24 hours after seeding using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. 1 pmol DNA was transfected per well for all assays unless otherwise indicated.

Mitochondrial Staining, Microscopy, and Image Analysis

Prior to live-cell imaging and mitochondrial staining, media in live cell chambers was replaced with phenol red-free DMEM (Invitrogen) for 1471.1 cells or phenol red-free RPMI (Invitrogen) for T47D and MCF-7 cells containing 10% charcoal-stripped fetal bovine serum (CS-FBS, Invitrogen). Cells were incubated with 250nM MitoTracker Red FM (Invitrogen) for 15 min at 37˚C and protected from light. Images were acquired as previously (*33*), using an Olympus IX71F fluorescence microscope (Scientific Instrument Company, Aurora, CO) with high-quality narrow band GFP filter (ex: HQ480/20 nm, em: HQ510/20 nm) and HQ:TRITC filter (ex: HQ545/30, em: HQ620/60) from Chroma Technology (Brattleboro, VT) with a 40X PlanApo oil immersion objective (NA 1.00) on an F-View Monochrome CCD camera. Images were analyzed for mitochondrial stain overlap with EGFP fusion constructs using ImageJ software and the JACoP plugin (*34*). JACoP was used to generate the colocalization statistic [i.e., Pearson's correlation coefficient (PCC) post Costes' automatic threshold algorithm (*35, 36*)]. PCC evaluates correlation between pairs of individual pixels from EGFP and MitoTracker stained cells. The higher the PCC value the higher the correlation. For

increased visual clarity of mitochondrial localization of the EGFP-fused constructs, spatial representations of pixel intensity correlation have been generated using Colocalization Colormap (ImageJ) (*37*).

Luciferase Assay

All constructs (3.5 μg of DNA) were co-transfected with 3.5 μg of p53-Luc Cis-Reporter (encoding for firefly luciferase, Agilent) in T47D and MCF-7 cells. To normalize for transfection efficiency, 0.35 μg of pRL-SV40 plasmid (encoding for renilla luciferase, gift from Dr. Philip Moos, University of Utah) was co-transfected. The Dual-Glo Luciferase Assay System (Promega, Madison, WI) was used to determine firefly luciferase activity and renilla luciferase per manufacturer's instructions. Luciferase activity was detected 24 hours after transfection using PlateLumino (Stratec Biomedical Systems, Birkenfeld, Germany). Firefly luciferase values were normalized for renilla luciferase. EGFP-p53 served as a positive control and EGFP as a negative control. The Dual-Glo Luciferase Assay was run three times independently, each in triplicate.

Caspase-9 Assay

T47D cells were probed 19 hours after transfection using CaspaLux®9-M_2D_2 kit (OncoImmunin, Inc., Gaithersburg, MD) per manufacturer's recommendations. The cells were then suspended in flow cytometry buffer (OncoImmunin, Inc.) and analyzed via the FACSAria-II (BD-BioSciences, University of Utah Core Facility) utilizing 488 nm (for EGFP) and 563 nm (for cleaved caspase 9 substrate) lasers. FACSDiva software was used as an evaluation tool. Only EGFP transfected cells at 507 nm emission were

analyzed. The samples were detected in the PE (phycoerythrin) channel with the 580 nm emission peak. Each construct was assayed three times (n=3).

TUNEL Assay

T47D cells were prepared 48 hours after transfection using In Situ Death Detection Kit, TMR red (Roche, Mannheim, Germany) per the company's protocol. The FACSAria-II was used to analyze the cells suspended in PBS (Invitrogen). The same FACS settings mentioned above with the caspase-9 assay were used. Only EGFP positive cells were analyzed for DNA segmentation. Each construct was analyzed three times (n=3).

Annexin-V Assay

At 48 hours post transfection, T47D cells were assayed for Annexin-V binding. The cells were suspended in 100 µl Annexin binding buffer (Invitrogen) and incubated with 5 µl Annexin-APC (Annexin-V conjugated to allophycocyanin, Invitrogen) for 15 minutes. The incubated cells were then diluted in 400 µl Annexin binding buffer and analyzed using the FACSCanto-II (BD-BioSciences, University of Utah Core Facility) with FACSDiva software. EGFP was excited at 488 nm wavelength and detected at 507 nm. APC was excited with 635 nm laser and detected at 660 nm. Analysis was based on EGFP positive cells. Each construct was tested three times (n=3).

7-AAD Assay

T47D and MCF-7 cells were stained with 7-aminoactinomycin D (7-AAD, Invitrogen) according to manufacturer's instructions 48 hours after transfection. The samples were analyzed using the FACSCanto-II (BD-BioSciences). Analyzed cells were gated for EGFP (as mentioned in Annexin-V assay). In addition, EGFP and 7-AAD were excited with the 488 nm laser. EGFP and 7-AAD were detected at 507 nm and 660 nm, respectively. Each construct was assayed three times (n=3).

Rescue Experiment Using Pifithrin-α

Six hours after transfection, T47D cells were incubated with 40 μM pifithrin-α (Cayman Chemical, Ann Arbor, MI) for 42 hours and compared to transfected cells without pifithrin-α. At the 48 hour time point, the 7-AAD assay was performed as above.

Rescue Experiment Using Pifithrin-μ

Six hours after transfection, T47D cells were incubated with 5 nM pifithrin-μ (Tocris Bioscience, Ellisville, MO) for 42 hours and compared to transfected cells without pifithrin-μ. At the 48 hour time point, the 7-AAD assay was performed as detailed above.

Rescue Experiment Using Bcl-XL

T47D cells were co-transfected with 1 pmol of MTS constructs and 1 pmol of pBcl-XL (Addgene). After 48 hours, cells were pelleted and assayed with 7-AAD as described above.

Statistical Analysis

All experiments were repeated in triplicate (n=3). The data were presented as the mean ± standard error. Statistical differences between each MTS-EGFP-p53 and its MTS-EGFP were resolved via unpaired t-test using GraphPad Prism software. The MTS-EGFP controls were compared to EGFP by one-way ANOVA with Tukey's posttest. A p value <0.05 was considered significant.

Results

Mitochondrial Localization of MTS-EGFP-p53

Mitochondrial targeting of all constructs was verified using fluorescence microscopy. Figure 5.2A shows 1471.1 cells, which are larger in size, spread well, and are generally easier to visualize versus T47D or MCF-7 cells. However, irrespective of the cell line, similar microscopy results were observed in T47D and MCF-7 cells (data not shown). PCC values range from +1 to -1; perfect correlation is represented by +1, anti-correlation by -1, and a PCC value of zero denotes random distribution (*34*). Following the example of Bolte and Cordelières, a PCC of 0.6 or greater will define colocalization, or co-compartmentalization (Figure 5.2B) (*34*). Fusing different MTSs to EGFP and p53 showed a high degree of colocalization with the mitochondria. EGFP-C1 served as negative control for colocalization analysis and as expected, there was no colocalization between EGFP alone and the mitochondria. EGFP and p53 tagged to TOM and XL targeted the mitochondria better than CCO-EGFP-p53 and OTC-EGFP-p53. CCO and OTC were the "weakest" MTSs since there was some nuclear targeting of both

A.

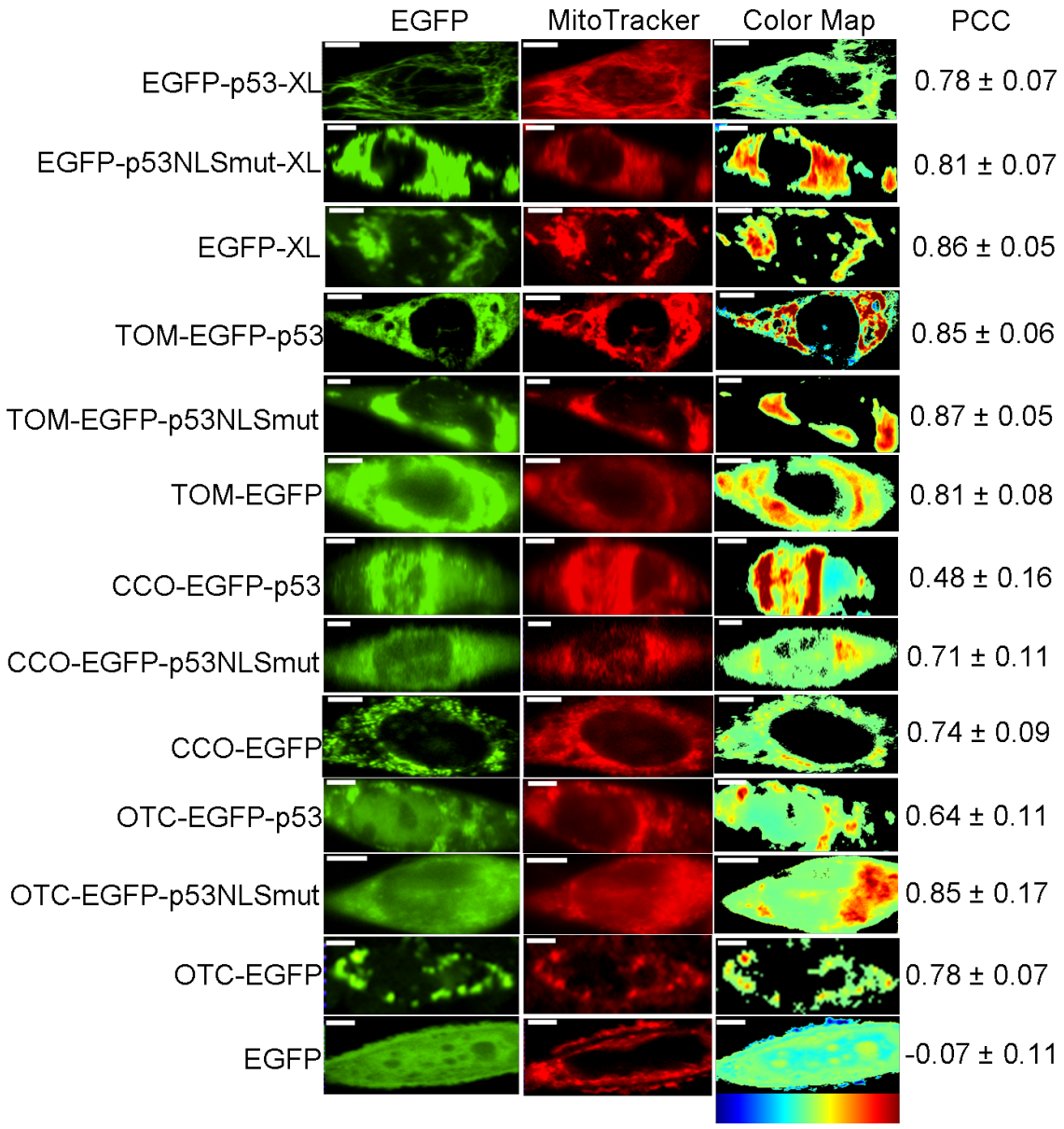


Figure 5.2. Colocalization of MTS constructs and MitoTracker Red mitochondrial stain in 1471.1 cells. A) Representative images of MTS-EGFP-p53, MTS-EGFP-p53 NLS mutation, MTS-EGFP and EGFP-C1 are shown in the left column with images of MitoTracker Red distribution in the middle column. The 'EGFP' and 'MitoTracker' columns have been false colored green and red, respectively. Enhanced visualization of

B.

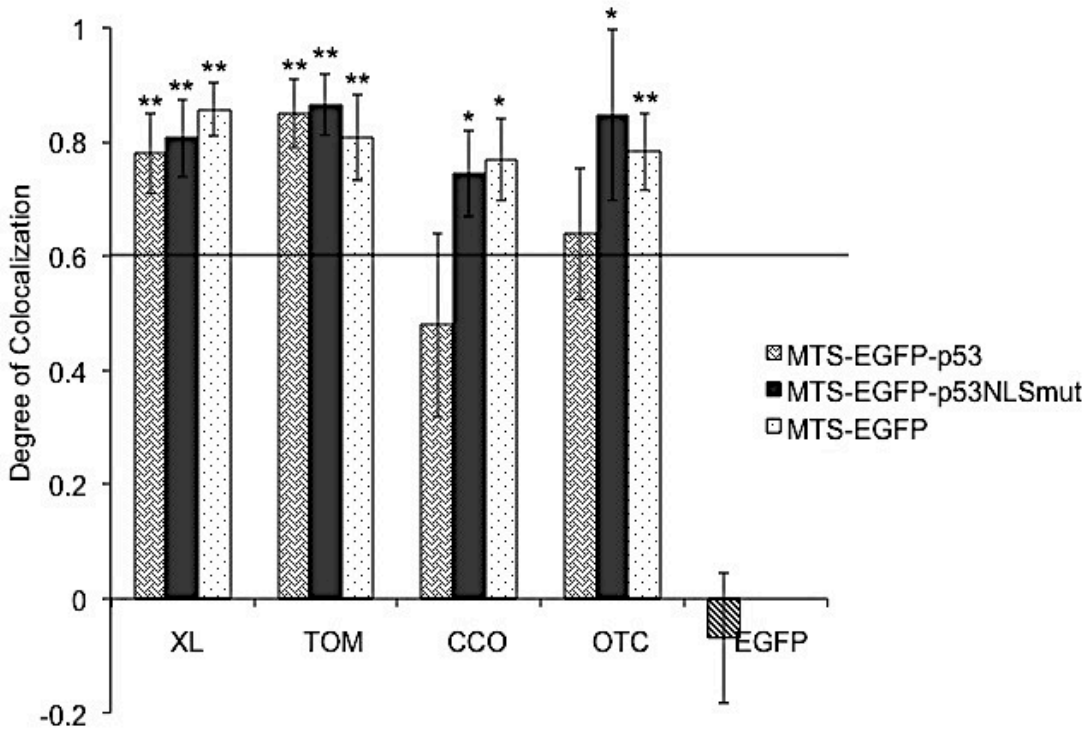


Figure 5.2 Cont. colocalized pixels is rendered in the 'Color Map' column. Warm colors depict pixels with highly correlated intensity and spatial overlap while cool colors are indicative of anti- or random correlation (colorbar for interpretation is shown below column). Corresponding PCC values are shown in the right column. White scale bars are all 10 μm. B) The degree of colocalization is represented by PCC following Costes' approach. All constructs with values higher than 0.6 are considered highly colocalized with mitochondrial stain MitoTracker Red. Statistical analysis was performed by using odds ratio. The adjusted odds ratio for PCC value of 0.6 were compared with each sample. *$p<0.05$, and **$p<0.01$ comparing odds ratio of lowest value for samples with odds ratio of 1 for PCC of 0.6.

CCO-EGFP-p53 and OTC-EGFP-p53. Therefore we mutated K319T and K320T in the nuclear localization signal (NLS) of p53 (*38*) which resulted in increased mitochondrial targeting for CCO-EGFP-p53 and OTC-EGFP-p53. For easier visualization of colocalization among constructs, a spatial depiction of pixel overlap and intensity correlation are provided in the 'Color Map' column (Figure 5.2A). The Color Map spectrum moves from cold to warm colors as pixel correlation increases (*37*).

Testing the Transcriptional Activity of MTS-EGFP-p53

To demonstrate the lack of transcriptional activity of these p53 constructs, a p53 reporter dual luciferase assay was performed in T47D (Figure 5.3A) and MCF-7 cells (Figure 5.3B). T47D cells contain a mutation in p53 (in the DBD which renders it inactive) that is also localized in the cytoplasm (*39, 40*) while MCF-7 cells express wild-type p53 mislocalized to the cytoplasm (*41*). TOM and XL fused to EGFP-p53 showed no nuclear activity in either cell lines. CCO-EGFP-p53 expressed similar transcriptional activity to EGFP-p53 (positive control) in T47D (Figure 5.3A) and significant activity in MCF-7 (Figure 5.3B) compared to the EGFP negative control. Introducing NLS mutations (K3319T and K320T) in CCO-EGFP-p53 resulted in major reduction of nuclear activity in both cell lines. OTC-EGFP-p53 showed low transcriptional activity in MCF-7 (Figure 5.3B) and a significant activation in T47D cells (Figure 5.3A). Surprisingly, introduction of NLS mutations into OTC-EGFP-p53 did not result in any changes in nuclear activity in either cell line (Figures 5.3A and B).

A.

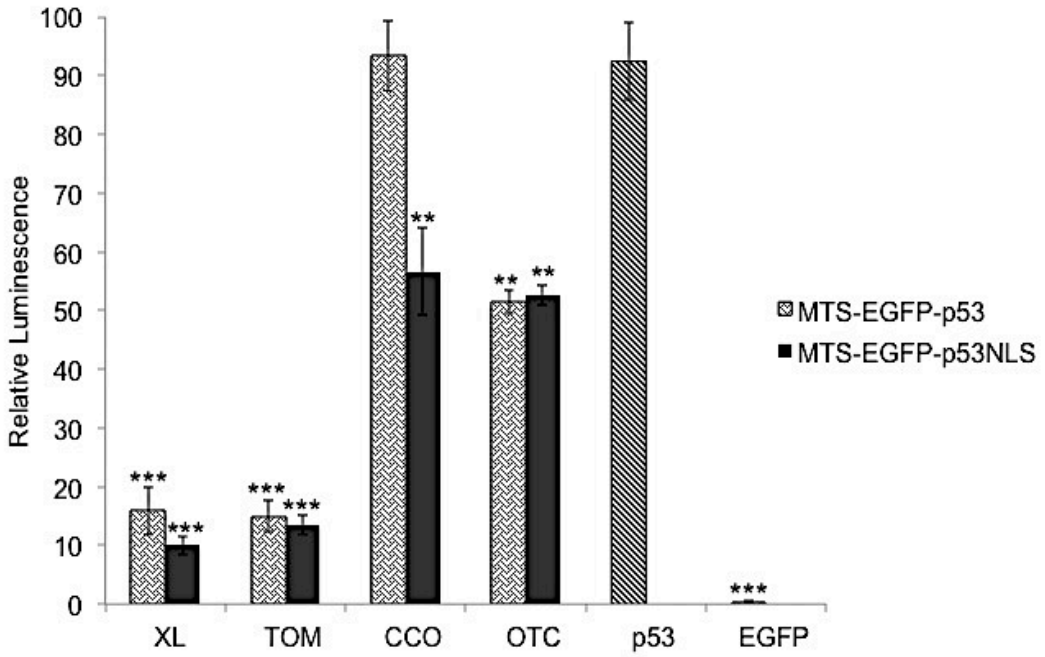


Figure 5.3. Luciferase assay: All MTS-EGFP-p53 and MTS-EGFP-p53 NLS mutation constructs were tested for their ability to activate a p53 reporter in A) T47D cells and B) MCF-7 cells. EGFP-p53 serves as a positive control and EGFP as a negative control. All constructs were corrected to EGFP-p53 control, which is set at 100%. Statistical analysis was performed by using one-way ANOVA with Tukey's posttest. **$p<0.005$ and ***$p<0.0005$ compared to EGFP-p53.

B.

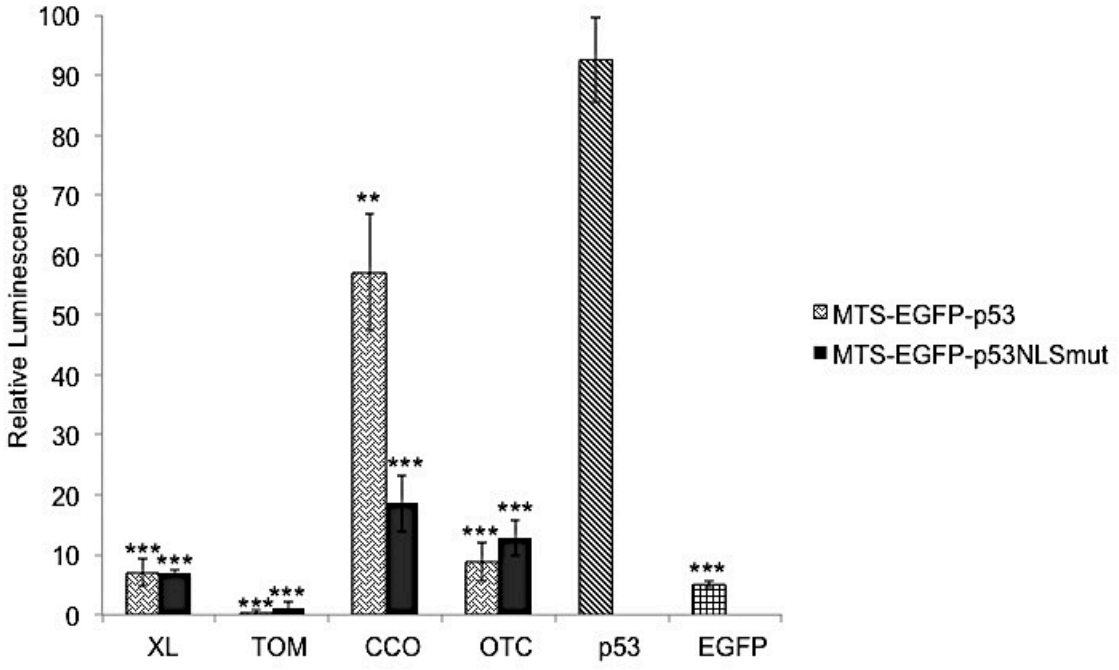


Figure 5.3 Cont.

mut

The Effect of MTS-EGFP-p53 on Early Apoptosis

In this paper we focused on T47D cell line because MCF-7 cells initiate apoptosis more rapidly than T47D (*42*). The apoptotic potential of p53 fused to different MTSs targeting the mitochondrial matrix, outer, and inner membranes was evaluated via caspase-9 assay, TUNEL assay, Annexin-V and 7-AAD. Caspases are a group of proteolytic enzymes that are directly involved in apoptosis by cleaving proteins such as lamin and PARP. Its inactive form procaspase-9 is activated through cytochrome c release and APAF-1 which occurs after mitochondrial outer membrane disruption (known as the intrinsic apoptotic pathway) (*43*). Caspase-9 itself cleaves the peptide sequence LEHD, which was used in the caspase-9 assay to measure the intrinsic apoptotic pathway (*44*). All MTS-EGFP-p53 constructs showed caspase-9 activity. However, only EGFP-p53-XL ($p<0.05$) and OTC-EGFP-p53 ($p<0.05$) were significantly different from their corresponding MTS-EGFP controls as shown in Figure 5.4.

The Effect of MTS-EGFP-p53 on DNA Fragmentation

Mid-stage apoptosis was then measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which detects DNA fragmentation by labeling the terminal end of nucleic acids. DNA fragmentation is a hallmark of apoptosis and is generated by caspase cleavage (*45*). Figure 5.5 illustrates that EGFP-p53-XL and CCO-EGFP-p53 are the only constructs that were statistically significant from their control MTS-EGFP. Constructs with mutations in the NLS of p53 did not differ from constructs without mutation (data not shown). Staurosporine, which activates caspase3/7 and leads to DNA fragmentation (*46*), served as a positive control.

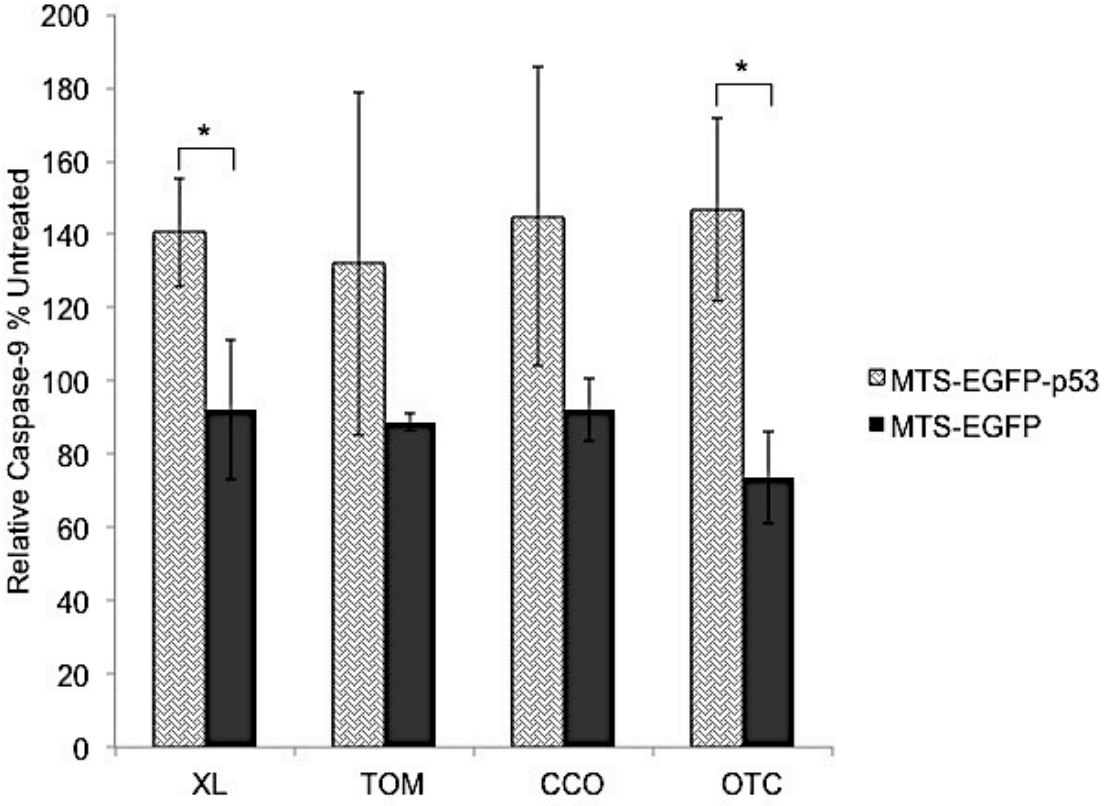


Figure 5.4. The activation of caspase-9 was analyzed 19 hours following transfection of T47D cells. All constructs were corrected to untreated control, which is set at 100%. Statistical analysis was performed by using unpaired t-test. * $p<0.05$ for MTS-EGFP-p53 compared to its MTS-EGFP negative control.

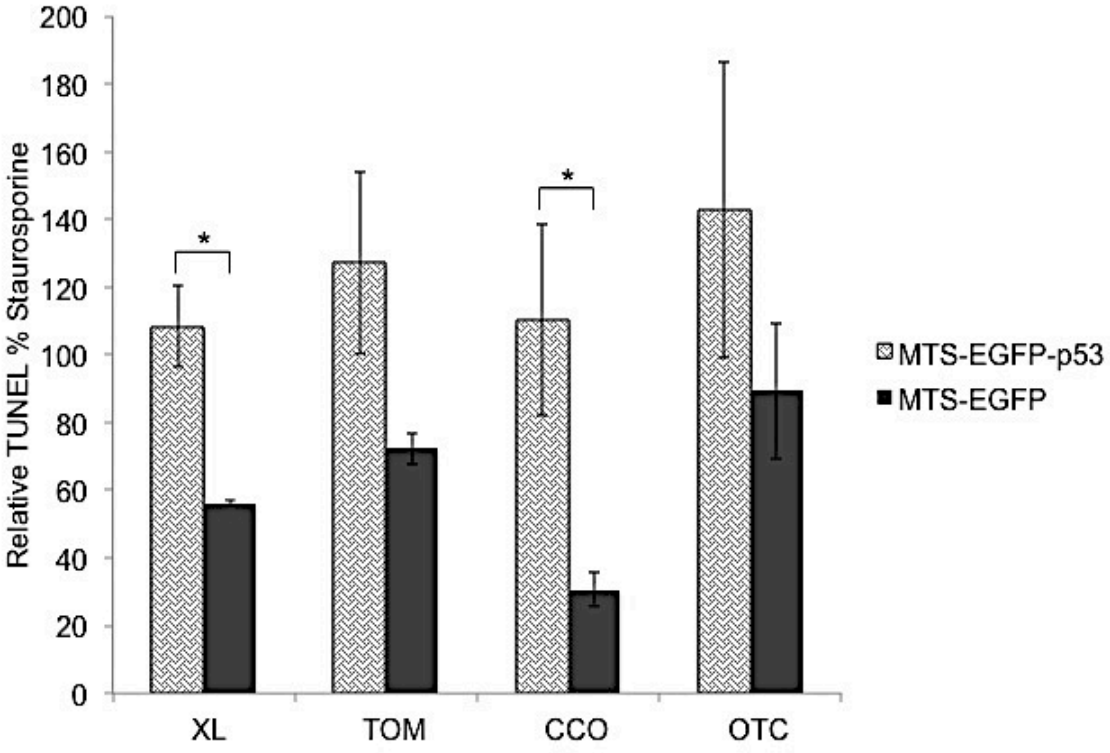


Figure 5.5. T47D cells were tested 48 hours following transfection. DNA fragmentation was analyzed with the TUNEL assay. All constructs were corrected to staurosporine positive control, which is set at 100%. Statistical analysis was performed by unpaired t-test. * $p<0.05$ for MTS-EGFP-p53 compared to its MTS-EGFP.

The Effect of MTS-EGFP-p53 on Plasma Membrane

The effects of the mitochondrial constructs were further explored by analyzing the induction of late apoptosis (Figure 5.6). Annexin-V was used to detect the externalization of phosphatidylserine on the cell surface of apoptotic cells via flow cytometry (*47, 48*). In the majority of healthy cells, the plasma membrane expresses phosphatidylserine on the cytosolic surface while in apoptotic cells, the phosphatidylserine is transported to the outer surface, which allows labeled Annexin-V to bind (*49*). All MTS-EGFP-p53 showed a significant effect on inducing late apoptosis than their corresponding MTS-EGFP controls in T47D cells (Figure 5.6). In addition, EGFP-XL was the only construct that showed similar activity to EGFP alone (Figure 5.6).

To further validate the effect of mitochondrial p53 on late apoptosis, the 7-AAD assay was performed via flow cytometry (Figure 5.7). The 7-AAD fluorescent marker cannot stain the DNA in healthy cells due to inability to penetrate an intact cell membrane (*50*). However, it is able to stain the DNA in apoptotic and necrotic cells because of their disrupted membrane (*51*). Similar to Annexin-V results, all mitochondrial p53 constructs showed significant 7-AAD intercalation with DNA compared to their MTS-EGFP controls (Figure 5.7A). In addition, EGFP-XL was the only construct that showed similar activity to EGFP alone (Figure 5.7A). In a cell line that is less resistant to apoptosis such as MCF-7 (*42*), EGFP-p53-XL is the only construct that was significant than its MTS-EGFP control (Figure 5.7B). In MCF-7, there was no statistical difference between p53 fused to TOM, CCO or OTC and their respective MTS-

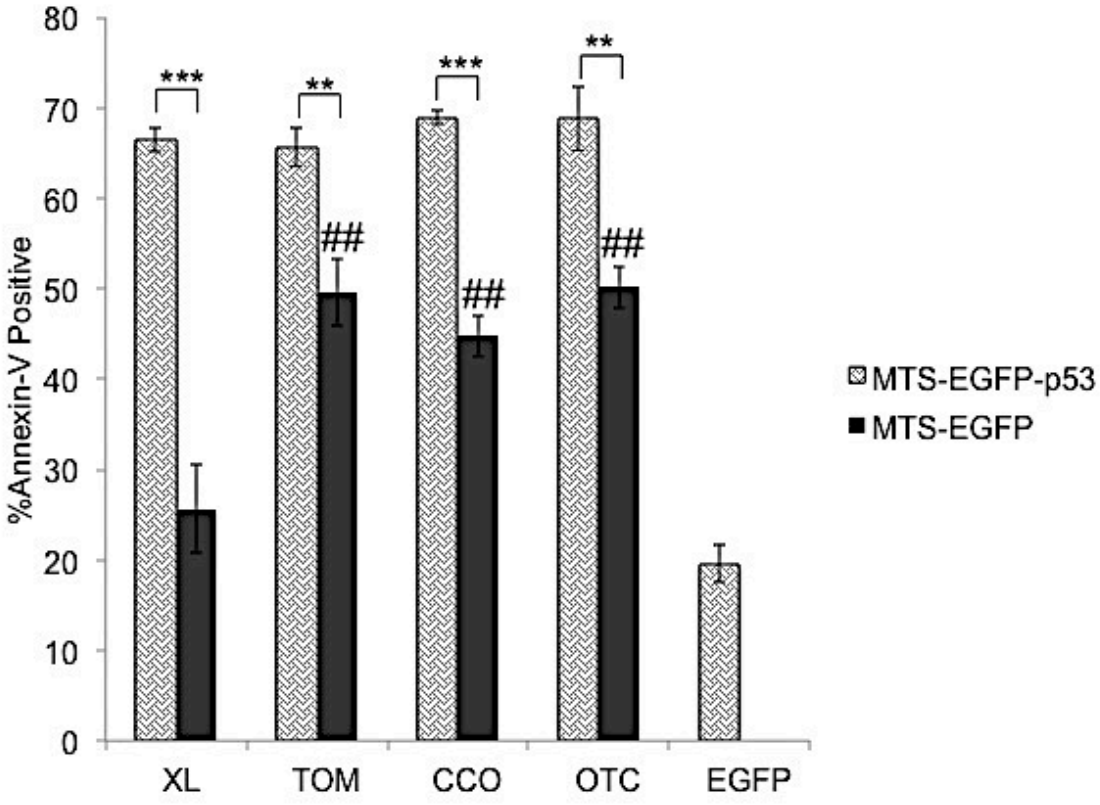


Figure 5.6. Annexin-V assay was conducted in T47D cells 48 hours after transfection. Statistical analysis was performed by one-way ANOVA with Tukey's posttest. **p<0.005 and ***p<0.0005 comparing MTS-EGFP-p53 to their MTS-EGFP controls. The negative controls (MTS-EGFP) were compared to EGFP-C1 using one-way ANOVA with Tukey's posttest ##p<0.005.

A.

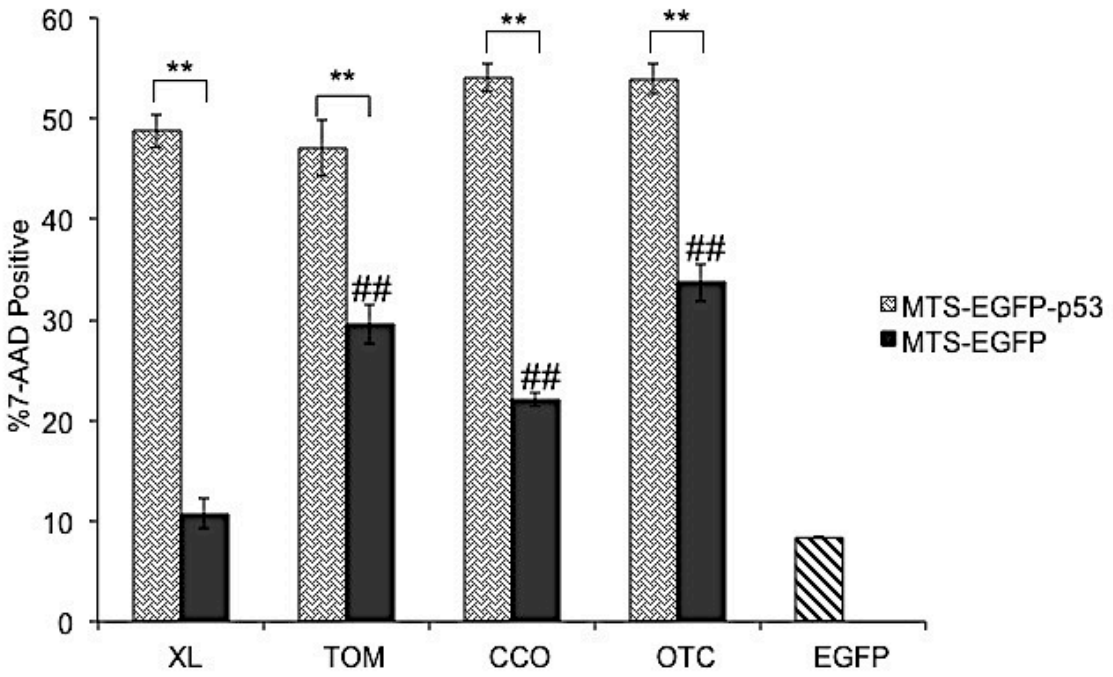


Figure 5.7. The 7-AAD assay was accomplished in (A) T47D and (B) MCF-7 cells 48 hours after transfection. Statistical analysis was performed by one-way ANOVA with Tukey's posttest. * $p<0.05$, **$p<0.005$ comparing MTS-EGFP-p53 to their MTS-EGFP controls. The controls (MTS-EGFP) were compared to EGFP-C1 using one-way ANOVA with Tukey's posttest #$p<0.05$, ###$p<0.0005$.

B.

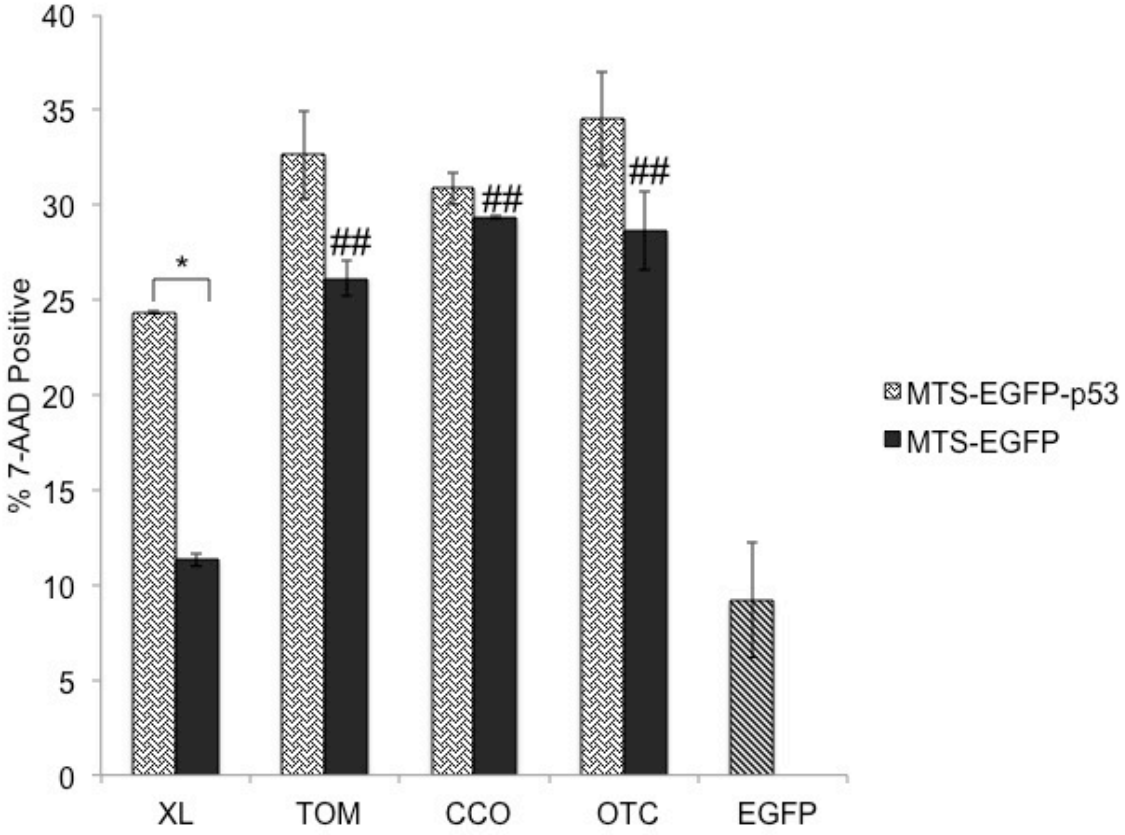


Figure 5.7 Cont.

EGFP controls (Figure 5.7B). Mutating the NLS of p53 in MTS-EGFP-p53 had no effect on 7-AAD permeation in both cell lines (data not shown). In addition, EGFP-XL showed the least significance from EGFP alone in MCF-7 (Figure 5.7B). Similar to Annexin V, EGFP-XL was also the only construct that did not show difference in activity to EGFP alone in T47D (Figure 5.7A).

Investigating the Apoptotic Mechanism of Mitochondrial p53 in T47D Cells

Apoptosis resulting from p53 transcriptional activity of our mitochondrial p53 constructs was examined via a pifithrin-α rescue experiment. Pifithrin-α is a small molecule, which inhibits p53-mediated transcriptional activity (*52, 53*). The effect of pifithrin-α was measured in 7-AAD assay (Figure 5.8A). The apoptotic effect of EGFP-p53 fused to either CCO or OTC was reduced significantly after pifithrin-α treatment. In addition, there was no impact on the apoptotic potential of EGFP-p53 fused to either XL or TOM (Figure 5.8A).

In order to investigate p53's apoptotic mechanism in the mitochondria, pifithrin-μ was used in a rescue experiment in the 7-AAD assay. Pifithrin-μ is a small molecule that reduces the binding affinity of p53 to the anti-apoptotic proteins, Bcl-2 and Bcl-XL (*54, 55*). Pifithrin-μ had a significant impact on the apoptotic potential of p53 fused to both XL and OTC (Figure 5.8B). However, apoptosis caused by p53 fused to either TOM or CCO was not rescued by pifithrin-μ. In addition, all the MTS-EGFP controls were not altered in this rescue experiment (data not shown).

A.

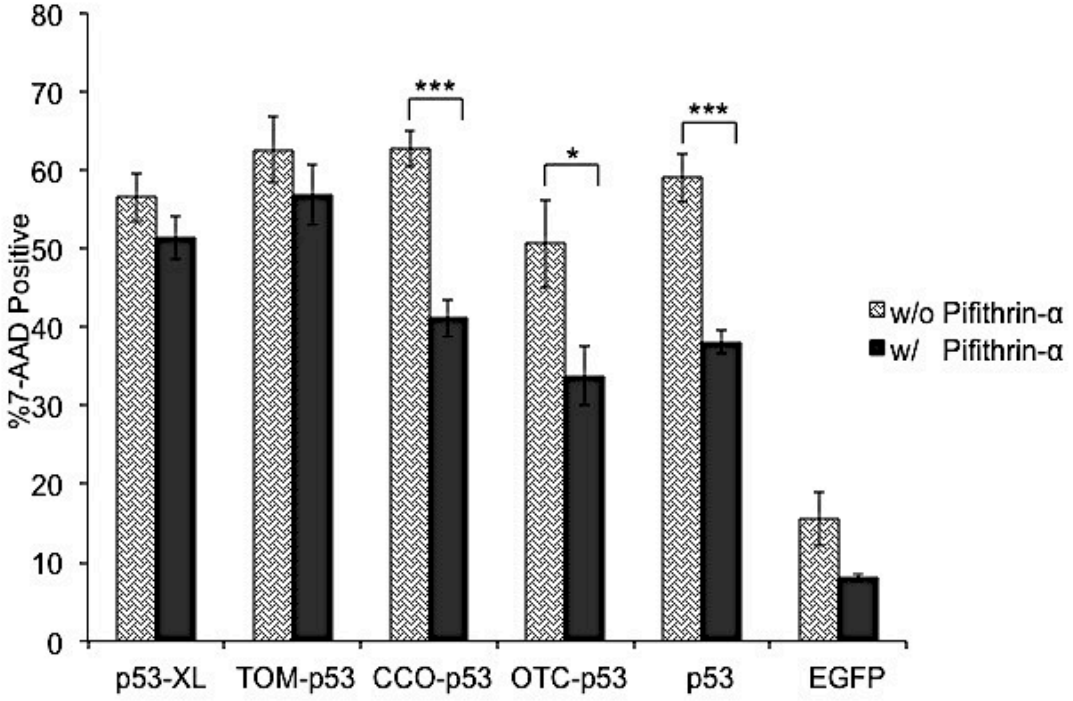
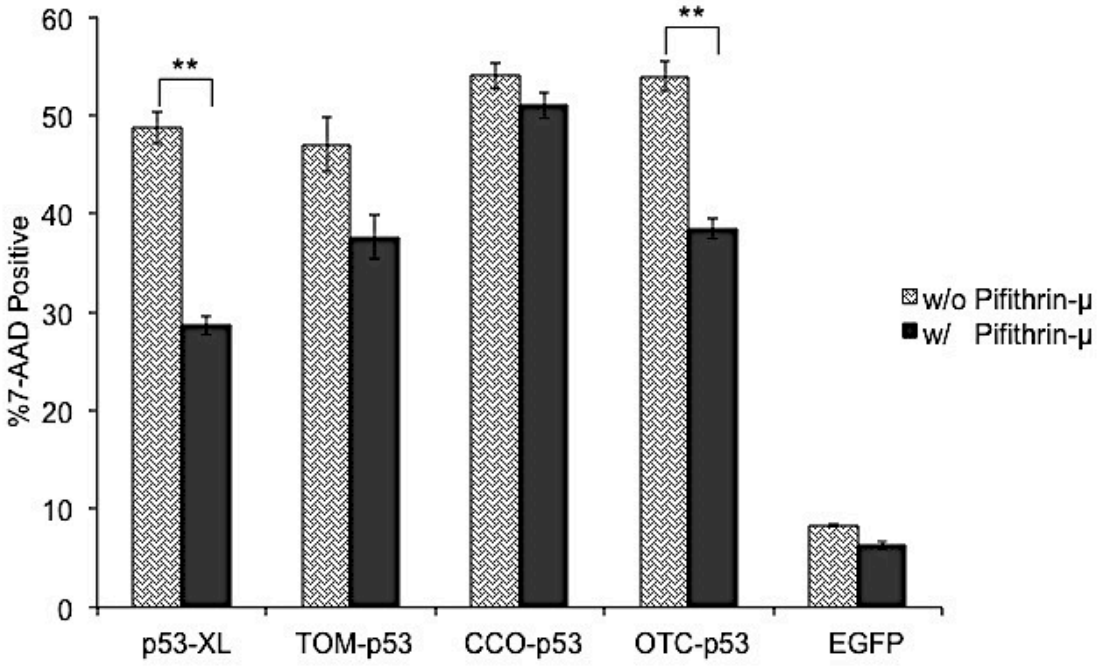


Figure 5.8. Rescue experiments using (A) pifithrin-α, (B) pifithrin-μ, or (C) Bcl-XL. 7-AAD assay was performed 48 hours after transfection in T47D cells. All constructs were fused to EGFP. Statistical analysis was performed by unpaired t-test. *$p<0.05$, and **$p<0.005$ comparing treated (with pifithrin-α, pifithrin-μ, or Bcl-XL) to untreated (no drug or Bcl-XL added).

B.



C.

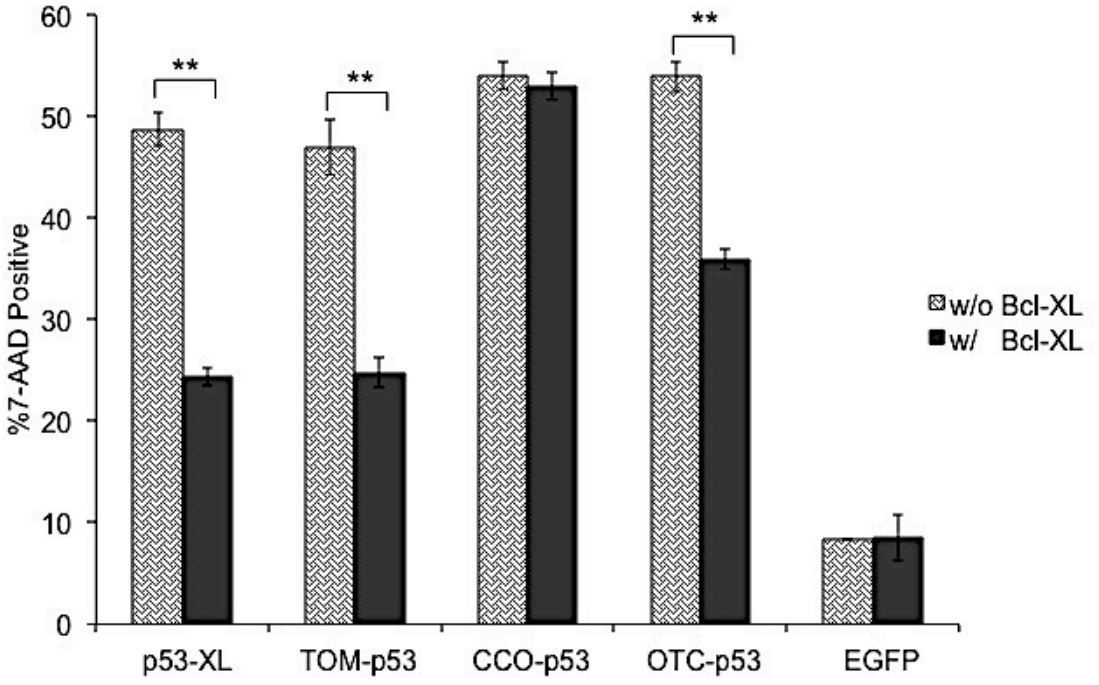


Figure 5.8 Cont.

The apoptotic mechanism was further explored by over-expression of the anti-apoptotic protein, Bcl-XL. The cells were then analyzed in the 7-AAD assay via flow cytometry. Bcl-XL had a significant effect on reducing 7-AAD positive cells treated with EGFP-p53 fused to TOM, XL, and OTC (Figure 5.8C). Cells treated with EGFP-p53-XL showed the most significant reduction in 7-AAD when co-transfected with Bcl-XL. Meanwhile, Bcl-XL had no effect on cells transfected with CCO-EGFP-p53.

Discussion

The tumor suppressor p53 was delivered to different mitochondrial sub-compartments in order to investigate its mitochondrial mechanism of apoptosis. We hypothesized that it might be possible that sending any protein (even EGFP) to mitochondria could be "toxic" to cells by disruption of mitochondrial function. Fusing p53 to MTSs targeting the mitochondrial outer membrane, inner membrane, and matrix did indeed result in apoptosis. However, we discovered that sending even EGFP to the mitochondrial matrix (OTC), the inner membrane (CCO), or the TOM complex (TOM) disturbed mitochondrial internal stability. Only p53-XL was capable of inducing mitochondrial apoptosis exclusively via mitochondrial p53 pathway.

Directing EGFP to the matrix and inner membrane via OTC and CCO, respectively, resulted in late stage apoptosis (7-AAD). This shows that fusing EGFP to OTC or CCO has a toxic effect on the mitochondria. We speculate that sending EGFP to the mitochondrial matrix and inner membrane could cause an imbalance in the sensitive mitochondrial system due to import through mitochondrial membranes. On the other

hand, targeting EGFP to the mitochondrial outer membrane using TOM and XL shows minimal toxicity with XL but significant toxicity with TOM. Since EGFP-XL is directed towards Bcl-XL (*56*) on the surface of the outer membrane (*26-28*), it is not expected to become imbedded into the mitochondrial membrane. On the other hand, the TOM-EGFP may interfere with TOM20 involved in mitochondrial import machinery (*57, 58*). The TOM complex is responsible for importing proteins across the mitochondrial outer membrane. TOM20 is one of the receptor subunits in the TOM complex (*59, 60*). It could be concluded that fusing any protein to the MTS from TOM20 might affect the sensitive import mechanism.

As p53 is a nuclear protein, the MTS fused to it will compete with the protein's nuclear localization signals (NLSs). p53 contains three NLSs; the most active of them is located at residues 305-322 (*38*). The nuclear import of large proteins is dependent on the availability of a NLS (*61-63*). To prevent the nuclear targeting of our constructs, we introduced mutations (K319T and K320T) in the strongest NLS of p53 (*38*). Colocalization data and p53 transcriptional activity assay showed an increase in mitochondrial targeting and a decrease in p53 nuclear activity after the introduction of the NLS mutations in CCO-EGFP-p53. According to our colocalization data, CCO-EGFP showed the lowest mitochondrial targeting compared to the other MTS-EGFP (Figure 5.2). The weak CCO signal explains the high transcriptional activity when fused to p53 without NLS mutations (Figure 5.3). The strong NLS in p53 competes with the relatively weak MTS from CCO and shifts the distribution to the nucleus. After mutating the strong NLS, the CCO MTS was also in competition with the other weak NLSs in p53 (*38*), which may explain why the CCO-EGFP-p53 NLS mutation still showed transcriptional

activity (Figure 5.3). However, the mutations did not have any effect on the mitochondrial targeting or nuclear activity of the TOM, XL, and OTC constructs. EGFP-p53 fused to TOM and XL showed minimal nuclear p53 activity presumably due to their strong mitochondrial signals. Introducing NLS mutations to p53 fused to TOM or XL did not show any reduction on the already low transcriptional activity (Figure 5.3). On the other hand, OTC-EGFP-p53 showed significant p53 nuclear activity but was not reduced upon NLS mutation (Figure 5.3).

In addition, the nuclear activity of MTS-EGFP-p53 differed between MCF-7 and T47D. These differences might be due to variability in proteins involved with p53 transcriptional activity, mitochondrial shuttling, or number of mitochondria in each cell line. In MCF-7, all MTS-EGFP-p53 constructs (with or without NLS mutations) showed minimum transcriptional activity except for the CCO-EGFP-p53, which had half the activity of wild type EGFP-p53 (Figure 5.3B). However, in T47D cells all MTS-EGFP-p53 constructs showed generally higher nuclear activity than in MCF-7, especially CCO-EGFP-p53, which showed the same nuclear activity as wild type EGFP-p53. CCO-EGFP-p53 NLS mutation and OTC-EGFP-p53 (with and without NLS mutation) showed fifty percent transcriptional activity in T47D (Figures 5.3A).

Even though the NLS mutations increased mitochondrial targeting of the CCO-EGFP-p53 construct, it did not have any effect on increasing the apoptotic potential. This was also the case for NLS mutations in all other constructs (data not shown). CCO-EGFP-p53 was significant compared to its CCO-EGFP control in TUNEL, Annexin-V and 7-AAD assays. Since CCO-EGFP showed cytotoxicity, the increase in apoptosis when attached to p53 was likely due to nuclear p53 activity. This is reflected in our

luciferase assay (Figure 5.3) and the rescue experiments with pifithrin-α, pifithrin-μ and Bcl-XL (Figure 5.8). The apoptotic activity of CCO-EGFP-p53 was reduced in the pifithrin-α (an inhibitor of p53 transcriptional activity) rescue experiment. However it was not rescued by either over-expression with the anti-apoptotic Bcl-XL or incubation with pifithrin-μ (an inhibitor of p53 binding to Bcl-2 and Bcl-XL) (*54, 55*). This demonstrates that CCO-EGFP-p53 does not initiate p53-mitochondrial-specific apoptosis (Figure 5.9).

OTC-EGFP-p53 also showed transcriptional activity. In addition, OTC-EGFP-p53 exhibited significant caspase-9 induction, and late stage apoptosis compared to its cytotoxic OTC-EGFP control. To examine if the increase of activity was due to nuclear or mitochondrial p53, the rescue experiments mentioned above were conducted and showed reduction in programmed cell death (Figure 5.8). This indicates that apoptosis was likely initiated through p53 binding to Bcl-XL and Bcl-2. In addition to the transcriptional activity data, this demonstrates that OTC-EGFP-p53 has activity in both the nucleus (rescued by pifithrin-α) and the mitochondria (rescued by pifithrin-μ and Bcl-XL) (Figure 5.9). Even though OTC directs p53 to the mitochondrial matrix, p53 is still able to interact with Bcl-XL and Bcl-2 proteins on the outer membrane. This could be due to cleavage of the MTS by endopeptidase, which enables p53 to target the outer membrane (*3, 12*).

Instead of targeting the protein to the matrix then translocating it to the outer membrane, as was the case for OTC, we fused EGFP-p53 to TOM to *directly* target the outer membrane. Direct targeting of the outer membrane with TOM-EGFP-p53 was able to initiate late stage apoptosis robustly compared to its TOM-EGFP control. Interestingly

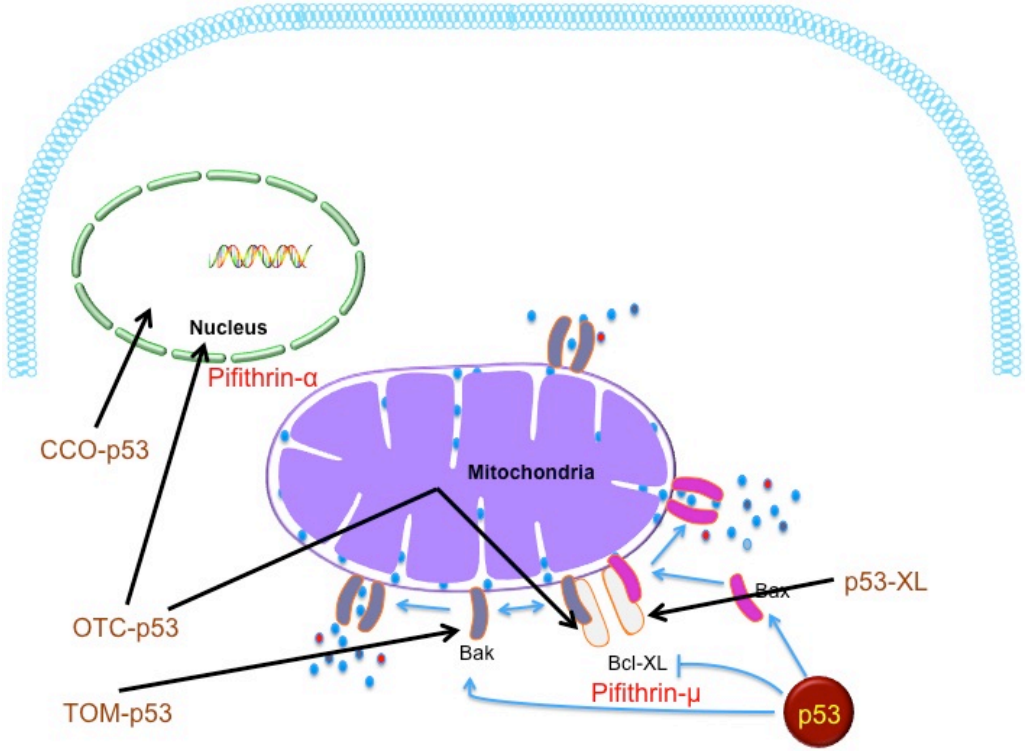


Figure 5.9. Speculated MTS-p53 apoptotic mechanism. p53-XL binds to Bcl-XL. TOM-p53 binds to Bak. OTC-p53 binds to Bcl-XL and shows transcriptional activity. CCO-p53 induces transcriptional activity. Pifithrin-α reduces the activity of both CCO-p53 and OTC-p53, while pifithrin-μ reduces the activity of OTC-p53 and p53-XL. In addition, overexpression of Bcl-XL reduces the activity of p53-XL, TOM-p53, and OTC-p53.

this increase in apoptosis was only rescued when Bcl-XL was co-transfected but not when pifithrin-α or pifithrin-μ was added (Figure 5.8). The pifithrin-α rescue experiment indicates that TOM-EGFP-p53 has no transcriptional activity. We speculate that TOM-EGFP-p53 is binding to pro-apoptotic Bak (Figure 5.9) and enhancing its oligomerization, which disrupts the mitochondrial outer membrane (*15, 16*). Bcl-XL forms a heterodimer with Bak and prevents Bak homodimerization (*16, 64, 65*). Therefore, when Bcl-XL is over-expressed, it competes with TOM-EGFP-p53 in binding with Bak and hence reduces apoptosis. Since pifithrin-μ reduces the binding of p53 to anti-apoptotic Bcl-XL and Bcl-2 and has no effect on binding to Bak, there was no reduction in programmed cell death.

In an effort to directly target the p53/Bcl-XL pathway, we fused XL to EGFP-p53. Directing p53 to the mitochondria via XL showed significant caspase-9, TUNEL, 7-AAD and Annexin-V activity compared to its EGFP-XL control. This apoptotic response was not due to transcriptional activity of p53 as shown in the luciferase assay data (Figure 5.3) and the pifithrin-α rescue experiment (Figure 5.8A). However, the apoptotic response was due to p53 direct interaction with anti-apoptotic Bcl-XL. To confirm this interaction, rescue experiments using pifithrin-μ and Bcl-XL were conducted and showed reduction in apoptosis (Figure 5.8B and C). In addition, the EGFP-XL control showed low toxicity compared to the other MTS-EGFP controls especially in MCF-7 cells (Figure 5.6 and 5.7). This data demonstrates that sending p53 to a specific protein (Bcl-XL) in the mitochondrial outer membrane causes p53 specific apoptosis. Table 5.2 is a summary of the results and speculation from this work.

Table 5.2. A summary of collected data and speculated mechanism. The table compares the four MTSs in localization, strength (*based on colocalization), mito-toxicity of MTS-EGFP, apoptotic response of MTS-EGFP-p53 compared to MTS-EGFP, and speculated apoptotic mechanism.

MTS	XL	TOM	CCO	OTC
Mitochondrial compartment	Outer surface of outer membrane	Outer membrane	Inner membrane	Matrix
Relative MTS Strength*	Strong	Strong	Weak	Medium/Strong
Intrinsic mito-toxicity of MTS-EGFP	Non-toxic	Toxic	Toxic	Toxic
p53 apoptotic response	Caspase-9, TUNEL, Annexin-V, and 7-AAD	Annexin-V, and 7-AAD	Annexin-V, and 7-AAD	Caspase-9, Annexin-V, and 7-AAD
Speculated apoptotic mechanism	Interacts with Bcl-XL	Interacts with Bak	Transcriptional p53	Transcriptional p5 and Interacts wit Bcl-XL

In summary, efficiency in targeting the mitochondria depends on the strength of the MTS. In the case of targeting proteins containing relatively strong NLSs such as the one found in p53 (residues 305-322) (*38*), mitochondrial targeting can only be achieved by using strong MTSs to counter the NLS. In our studies, relatively weaker MTSs are not efficient enough to compete with the strong NLS in p53. In addition, protein targeting to the mitochondria disrupts the sensitive balance in the mitochondria, which initiates intrinsic apoptosis. Except for EGFP-XL, all mitochondrial constructs had apoptotic effects. We conclude that EGFP-p53-XL was the most specific to p53/Bcl-XL mitochondrial pathway. Our data shows that not all mitochondrial targeting signals are optimal for mitochondrial induction of apoptosis with p53. In conclusion, specific binding of p53 to mitochondrial Bcl-XL (and hence apoptotic activity) is best achieved by directly targeting p53 to Bcl-XL via the XL MTS. This work therefore provides a mechanistic understanding of mitochondrial p53 apoptosis. Ultimately, p53-XL gene therapy is expected to be beneficial for other types of progressive cancers that currently have no effective therapy.

Acknowledgements

This work was funded by NIH R01-CA151847. We acknowledge Katharina Opper, Geoff Miller, Andy Dixon, Ben Bruno, Rian Davis, David Woessner, and Shams Reaz for scientific discussions.

References

1. Erster, S., Mihara, M., Kim, R. H., Petrenko, O., and Moll, U. M. (2004) In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation, *Mol. Cell Biol. 24*, 6728-6741.

2. Erster, S., and Moll, U. M. (2005) Stress-induced p53 runs a transcription-independent death program, *Biochem. Biophys. Res. Commun. 331*, 843-850.

3. Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) p53 has a direct apoptogenic role at the mitochondria, *Mol. Cell 11*, 577-590.

4. Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis, *Science 303*, 1010-1014.

5. Zhao, Y., Chaiswing, L., Velez, J. M., Batinic-Haberle, I., Colburn, N. H., Oberley, T. D., and St Clair, D. K. (2005) p53 translocation to mitochondria precedes its nuclear translocation and targets mitochondrial oxidative defense protein-manganese superoxide dismutase, *Cancer Res. 65*, 3745-3750.

6. Canugovi, C., Maynard, S., Bayne, A. C., Sykora, P., Tian, J., de Souza-Pinto, N. C., Croteau, D. L., and Bohr, V. A. (2010) The mitochondrial transcription factor A functions in mitochondrial base excision repair, *DNA Repair 9*, 1080-1089.

7. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Surfing the p53 network, *Nature 408*, 307-310.

8. el-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., and et al. (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis, *Cancer Res. 54*, 1169-1174.

9. Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops, *Oncogene 24*, 2899-2908.

10. Miyashita, T., and Reed, J. C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, *Cell 80*, 293-299.

11. Vousden, K. H., and Lane, D. P. (2007) p53 in health and disease, *Nat. Rev. Mol. Cell Biol 8*, 275-283.

12. Marchenko, N. D., Zaika, A., and Moll, U. M. (2000) Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling, *J. Biol. Chem. 275*, 16202-16212.

13. Tomita, Y., Marchenko, N., Erster, S., Nemajerova, A., Dehner, A., Klein, C., Pan, H., Kessler, H., Pancoska, P., and Moll, U. M. (2006) WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization, *J. Biol. Chem. 281*, 8600-8606.

14. Chipuk, J. E., Maurer, U., Green, D. R., and Schuler, M. (2003) Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription, *Cancer Cell 4*, 371-381.

15. Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex, *Nat. Cell Biol. 6*, 443-450.

16. Pietsch, E. C., Leu, J. I., Frank, A., Dumont, P., George, D. L., and Murphy, M. E. (2007) The tetramerization domain of p53 is required for efficient BAK oligomerization, *Cancer Biol. Ther. 6*, 1576-1583.

17. Green, D. R., and Reed, J. C. (1998) Mitochondria and apoptosis, *Science 281*, 1309-1312.

18. Reed, J. C. (1997) Cytochrome c: can't live with it--can't live without it, *Cell 91*, 559-562.

19. Green, D. R., and Evan, G. I. (2002) A matter of life and death, *Cancer Cell 1*, 19-30.

20. Wolff, S., Erster, S., Palacios, G., and Moll, U. M. (2008) p53's mitochondrial translocation and MOMP action is independent of Puma and Bax and severely disrupts mitochondrial membrane integrity, *Cell Res. 18*, 733-744.

21. Brenner, C., Le Bras, M., and Kroemer, G. (2003) Insights into the mitochondrial signaling pathway: what lessons for chemotherapy?, *J. Clin. Immunol. 23*, 73-80.

22. von Heijne, G. (1986) Mitochondrial targeting sequences may form amphiphilic helices, *EMBO J. 5*, 1335-1342.

23. Koehler, C. M. (2004) New developments in mitochondrial assembly, *Annu. Rev. Cell Dev. Biol. 20*, 309-335.

24. Roise, D., and Schatz, G. (1988) Mitochondrial presequences, *J. Biol. Chem. 263*, 4509-4511.

25. Wiedemann, N., Frazier, A. E., and Pfanner, N. (2004) The protein import machinery of mitochondria, *J. Biol. Chem. 279*, 14473-14476.

26. Kaufmann, T., Schlipf, S., Sanz, J., Neubert, K., Stein, R., and Borner, C. (2003) Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane, *J. Cell Biol. 160*, 53-64.

27. Pollack, M., and Leeuwenburgh, C. (2001) Apoptosis and aging: role of the mitochondria, *J. Gerontol. 56*, B475-482.

28. Lindsay, J., Esposti, M. D., and Gilmore, A. P. (2011) Bcl-2 proteins and mitochondria--specificity in membrane targeting for death, *Biochim. Biophys. Acta. 1813*, 532-539.

29. Rehling, P., Brandner, K., and Pfanner, N. (2004) Mitochondrial import and the twin-pore translocase, *Nat. Rev. Mol. Cell Biol. 5*, 519-530.

30. Power, S. D., Lochrie, M. A., Patterson, T. E., and Poyton, R. O. (1984) The nuclear-coded subunits of yeast cytochrome c oxidase. II. The amino acid sequence of subunit VIII and a model for its disposition in the inner mitochondrial membrane, *J. Biol. Chem. 259*, 6571-6574.

31. Fabrizi, G. M., Sadlock, J., Hirano, M., Mita, S., Koga, Y., Rizzuto, R., Zeviani, M., and Schon, E. A. (1992) Differential expression of genes specifying two isoforms of subunit VIa of human cytochrome c oxidase, *Gene 119*, 307-312.

32. Isaya, G., Fenton, W. A., Hendrick, J. P., Furtak, K., Kalousek, F., and Rosenberg, L. E. (1988) Mitochondrial import and processing of mutant human ornithine transcarbamylase precursors in cultured cells, *Mol. Cell Biol. 8*, 5150-5158.

33. Dixon, A. S., Kakar, M., Schneider, K. M., Constance, J. E., Paullin, B. C., and Lim, C. S. (2009) Controlling subcellular localization to alter function: Sending oncogenic Bcr-Abl to the nucleus causes apoptosis, *J. Control. Release 140*, 245-249.

34. Bolte, S., and Cordelieres, F. P. (2006) A guided tour into subcellular colocalization analysis in light microscopy, *J. Microscopy 224*, 213-232.

35. Costes, S. V., Daelemans, D., Cho, E. H., Dobbin, Z., Pavlakis, G., and Lockett, S. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells, *Biophys. J. 86*, 3993-4003.

36. Adler, J., and Parmryd, I. Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient, *Cytometry A 77*, 733-742.

37. Jaskolski, F., Mulle, C., and Manzoni, O. J. (2005) An automated method to quantify and visualize colocalized fluorescent signals, *J. Neurosci. Meth. 146*, 42-49.

38. Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A., and Rotter, V. (1990) Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis, *Mol. Cell Biol. 10*, 6565-6577.

39. Schafer, J. M., Lee, E. S., O'Regan, R. M., Yao, K., and Jordan, V. C. (2000) Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice, *Clin. Cancer Res. 6*, 4373-4380.

40. Alkhalaf, M., and El-Mowafy, A. M. (2003) Overexpression of wild-type p53 gene renders MCF-7 breast cancer cells more sensitive to the antiproliferative effect of progesterone, *J. Endocrinol. 179*, 55-62.

41. Takahashi, K., Sumimoto, H., Suzuki, K., and Ono, T. (1993) Protein synthesis-dependent cytoplasmic translocation of p53 protein after serum stimulation of growth-arrested MCF-7 cells, *Mol. Carcinog. 8*, 58-66.

42. Mooney, L. M., Al-Sakkaf, K. A., Brown, B. L., and Dobson, P. R. (2002) Apoptotic mechanisms in T47D and MCF-7 human breast cancer cells, *British J. Cancer 87*, 909-917.

43. Chowdhury, I., Tharakan, B., and Bhat, G. K. (2008) Caspases - an update, *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 151*, 10-27.

44. Yin, Q., Park, H. H., Chung, J. Y., Lin, S. C., Lo, Y. C., da Graca, L. S., Jiang, X., and Wu, H. (2006) Caspase-9 holoenzyme is a specific and optimal procaspase-3 processing machine, *Mol. Cell 22*, 259-268.

45. Loo, D. T., and Rillema, J. R. (1998) Measurement of cell death, *Meth. Cell Biol. 57*, 251-264.

46. Mooney, L. M., Al-Sakkaf, K. A., Brown, B. L., and Dobson, P. R. (2002) Apoptotic mechanisms in T47D and MCF-7 human breast cancer cells, *Br. J. Cancer 87*, 909-917.

47. Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., and van Oers, M. H. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis, *Blood 84*, 1415-1420.

48. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine

expression on early apoptotic cells using fluorescein labelled Annexin V, *J. Immunol. Meth. 184*, 39-51.

49. Metkar, S. S., Wang, B., Catalan, E., Anderluh, G., Gilbert, R. J., Pardo, J., and Froelich, C. J. (2011) Perforin rapidly induces plasma membrane phospholipid flip-flop, *PLoS One 6*, e24286.

50. Schmid, I., Krall, W. J., Uittenbogaart, C. H., Braun, J., and Giorgi, J. V. (1992) Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry, *Cytometry 13*, 204-208.

51. Serrano, M. J., Sanchez-Rovira, P., Algarra, I., Jaen, A., Lozano, A., and Gaforio, J. J. (2002) Evaluation of a gemcitabine-doxorubicin-paclitaxel combination schedule through flow cytometry assessment of apoptosis extent induced in human breast cancer cell lines, *Jap. J. Cancer Res. 93*, 559-566.

52. Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy, *Science 285*, 1733-1737.

53. Liu, X., Chua, C. C., Gao, J., Chen, Z., Landy, C. L., Hamdy, R., and Chua, B. H. (2004) Pifithrin-alpha protects against doxorubicin-induced apoptosis and acute cardiotoxicity in mice, *Am. J. Physiol. 286*, H933-939.

54. Hagn, F., Klein, C., Demmer, O., Marchenko, N., Vaseva, A., Moll, U. M., and Kessler, H. (2010) BclxL changes conformation upon binding to wild-type but not mutant p53 DNA binding domain, *J. Biol. Chem. 285*, 3439-3450.

55. Strom, E., Sathe, S., Komarov, P. G., Chernova, O. B., Pavlovska, I., Shyshynova, I., Bosykh, D. A., Burdelya, L. G., Macklis, R. M., Skaliter, R., Komarova, E. A., and Gudkov, A. V. (2006) Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation, *Nat. Chem. Biol. 2*, 474-479.

56. Vaseva, A. V., and Moll, U. M. (2009) The mitochondrial p53 pathway, *Biochim. Biophys. Acta. 1787*, 414-420.

57. Schmidt, O., Pfanner, N., and Meisinger, C. (2010) Mitochondrial protein import: from proteomics to functional mechanisms, *Nat. Rev. Mol. Cell Biol. 11*, 655-667.

58. Mossalam, M., Dixon, A. S., and Lim, C. S. (2010) Controlling subcellular delivery to optimize therapeutic effect, *Ther. Deliv. 1*, 169-193.

59. Endo, T., and Kohda, D. (2002) Functions of outer membrane receptors in mitochondrial protein import, *Biochim. Biophys. Acta. 1592*, 3-14.

60. Dolezal, P., Likic, V., Tachezy, J., and Lithgow, T. (2006) Evolution of the molecular machines for protein import into mitochondria, *Science 313*, 314-318.

61. Mattaj, I. W., and Englmeier, L. (1998) Nucleocytoplasmic transport: the soluble phase, *Annu. Rev. Biochem. 67*, 265-306.

62. Gorlich, D., and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm, *Annu. Rev. Cell Dev. Biol. 15*, 607-660.

63. Davis, J. R., Kakar, M., and Lim, C. S. (2007) Controlling protein compartmentalization to overcome disease, *Pharm. Res. 24*, 17-27.

64. Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis, *Science 275*, 983-986.

65. Galluzzi, L., Morselli, E., Kepp, O., Tajeddine, N., and Kroemer, G. (2008) Targeting p53 to mitochondria for cancer therapy, *Cell Cycle 7*, 1949-1955.

CHAPTER 6

DESIGNING A PROTEIN SWITCH TO CONTROL PROTEASOMAL TARGETING

Abstract

Proteasomal degradation of proteins is necessary especially in neurodegenerative diseases and cancer. The tumor suppressor p53 is one of the proteins that is constantly degraded in unstressed and some cancer cells. The degradation of p53 is due to binding to the ubiquitin ligase, MDM2, which is overexpressed in a variety of human cancers. In this work, a proteasomal protein switch was constructed taking advantage of p53 degradation. p53 was fused to EGFP, a nuclear localization signal, a nuclear export signal, and a ligand binding domain. We provide indirect evidence that the resulted fused protein translocated from the cytoplasm to the proteasome upon the addition of a ligand. This approach could be used to capture and degrade cytoplasmic oncogenic proteins via fusing a binding domain. It also gives an alternative method for p53/MDM2 inhibitor screening using fluorescence microscopy.

Introduction

Proteins are targeted to the proteasome for degradation, when marked with a death signal (polyubiquitin chain) (*1*). Proteasomal targeting of proteins could be utilized

for neurodegenerative diseases and cancer (*2, 3*). Knowledge of the ubiquitin-proteasome system is pivotal for understanding the degradation of proteins via the proteasome (*4*). A protein that is destined for degradation goes through a post-translational modification called ubiquitylation. Ubiquitylation is a process that covalently modifies proteins with ubiquitin via the enzymatic activity of an E1, an E2, and an E3 protein (Figure 6.1). Ubiquitin is first activated and transferred to the E1 active cysteine residue via a thioester linkage in a process requiring ATP. Ubiquitin is then transferred to the active cysteine residue of an ubiquitin-conjugating enzyme, E2, via a trans(thio)esterification reaction. Ubiquitin ligase, E3, interacts with both E2 and the targeted protein. In general, E3 is important for mediating substrate specificity (*241*). Subsequently, ubiquitin is ligated to the ε-amino group of a lysine of the target protein through a more stable isopeptide linkage. Since ubiquitin itself contains seven lysine residues, it allows the synthesis of polyubiquitin chains though isopeptide linkages. The process is then repeated giving rise to a polyubiquitin chain, which is recognized by the 19S regulatory caps of the proteasome. In the case of p53 degradation, MDM2 acts as the ubiquitin ligase (E3) while Ubc5 is the ubiquitin conjugating enzyme (E2) (*5*). There are six lysine residues in p53 that get ubiquitinated by MDM2: K370, K372, K373, K381, K381, and K386 (*6*).

The tumor suppressor p53 is a transcription factor that is found at low levels in normal unstressed cells through ubiquitylation and proteasomal degradation (*7, 8*). The half-life of p53 is 6-20 minutes in healthy cells. However the concentration of p53 is increased 3- to10-fold and the half-life is improved to hours in response to a variety of stresses including DNA damage, hyperproliferation, and hypoxia (*9, 10*). When p53 is induced, certain genes are up-regulated promoting growth arrest, apoptosis, and DNA

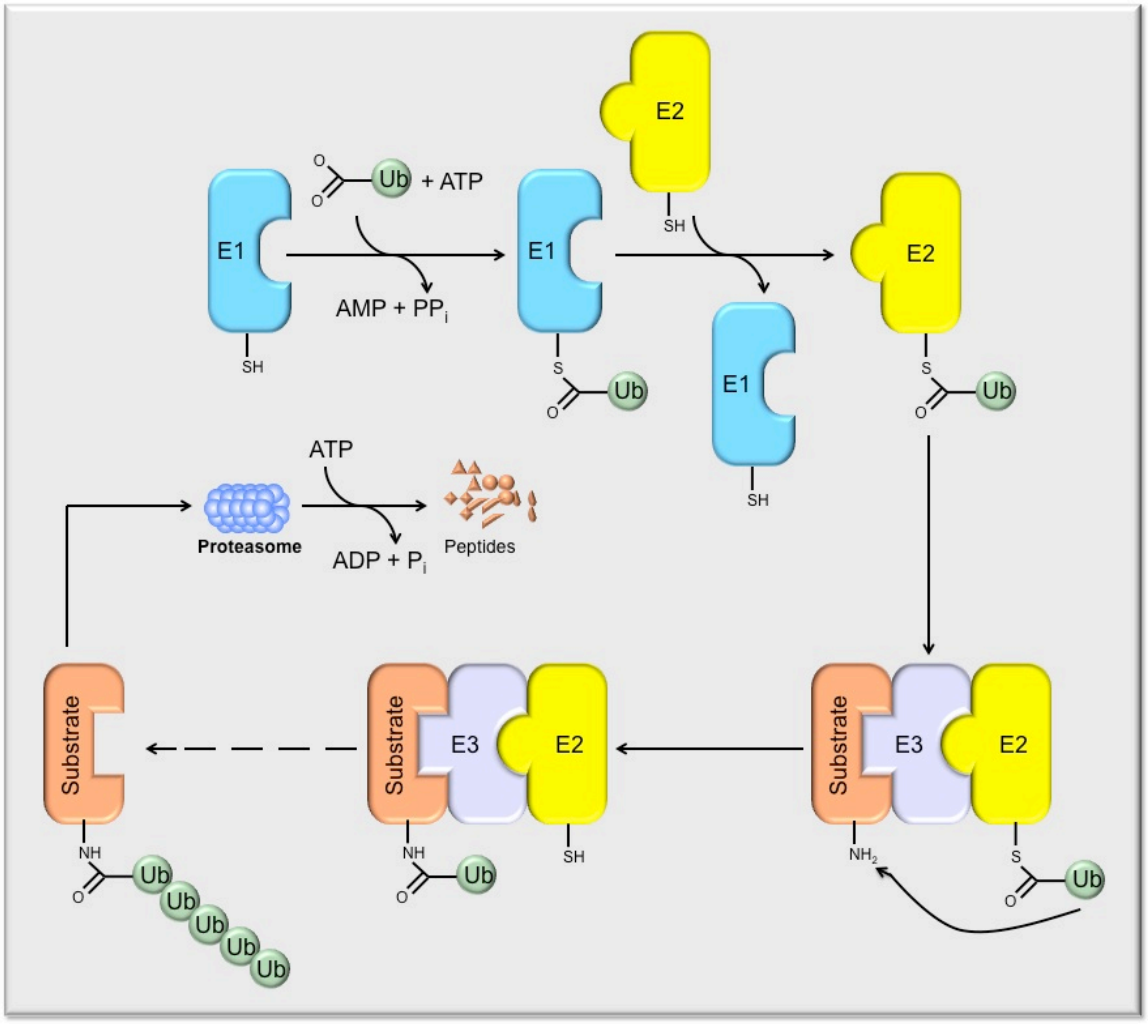


Figure 6.1. The enzymes and reactions of the ubiquitin-proteasome system. Ubiquitin is transferred to E1 enzyme in an ATP-dependent manner. Ubiquitin is then transferred to E2 enzyme, which carries the ubiquitin to E3 enzyme. The substrate (targeted protein) is ubiquitinated upon E3 binding. The ubiquitination of the substrate is repeated, which leads to the formation of polyubiquitin. The proteasome recognizes the polyubiquitin and initiates the degradation of the substrate.

repair (*11*). Loss of p53 activity predisposes cells to cancer, while errant p53 activation can lead to premature senescence and apoptosis. MDM2, which is also known as HDM2 in human, is one of the negative regulators of p53 (*12*). In addition, p53 regulates and induces the expression of MDM2 creating a negative feedback loop (*13*).

MDM2 contains a p53 binding domain, nuclear localization signal, nuclear export signal, and a RING finger domain. When p53 is activated, MDM2 binds to p53 to inhibit its transcriptional activity, and to mark it for proteasomal degradation (*14, 15*). Degradation of p53 is dependent on the nuclear exclusion of p53 by MDM2 (*16*). However, some nuclear proteasomal degradation of p53 can be induced by MDM2 (*17*). In addition, MDM2 is capable of regulating its own levels through auto-ubiquitination. There is an overexpression of MDM2 in a variety of human cancers such as breast cancer, melanoma, esophageal cancer, leukemia, sarcoma, non-small cell lung cancer, and non-Hodgkin's lymphoma (*18*). Overexpression of MDM2 is correlated with poor diagnosis in glioma, leukemia, and sarcoma (*19*). Preclinical studies have focused on inhibiting MDM2 to restore p53 activity in cancer cells (*20-22*). Since the MDM2-p53 interaction has been well characterized, small molecule inhibitors of MDM2 has been developed (*23, 24*). Nutlin-3 is one the most commonly used inhibitor, which targets MDM2 by interacting with the p53-binding pocket of MDM2, prevent its binding to p53, and hence increase the transcriptional activity of p53 (*25, 26*).

In this work, we took advantage of our nuclear protein switch (PS), which contains a ligand inducible nuclear import (*27*) and an export signal to control the localization to the nucleus (*28*). The localization is controlled by a dexamethasone-specific ligand-binding domain (LBD), which was cloned from a nuclear receptor, the

glucocorticoid receptor (GR). When expressed, the protein is localized to the cytoplasm but is targeted to the nucleus upon ligand induction (*29-31*).

In an effort to create a proteasomal protein switch, we fused p53 to the nuclear protein switch. Upon ligand addition, the p53 is localized to the nucleus where it binds to MDM2. This binding allows the ubiquitination of p53 and further proteasomal targeting.

Materials and Methods

Construction of EGFP-PS-p53

The DNA encoding p53 was amplified through PCR from pCMV-p53 wt (a generous gift from Dr. S. J. Baker, Addgene, Cambridge, MA) using the primers 5′-GCGCGCGGATCCGCCATGGAGGAGCCGCAGT-3′ and 5′-GCGCGCGGATCCT-CAGTCTGAGTCAGGCCCTTCTGTC-3′. This was subcloned into the BamHI restriction enzyme site in the EGFP-HIV-MycA8-GRLBD (EGFP-PS) plasmid constructed previously (*30*). The EGFP-PS contains a nuclear export signal (NES) from HIV-rev protein (*29*), a nuclear localization signal (NLS) from MycA8 protein (*30*) and a ligand binding domain from glucocorticoid receptor (GRLBD) with a point mutation (C656G) that makes it 10 times more sensitive to the agonist dexamethasone (dex) (*32*).

Construction of E-PS-p53 ΔMBD

p53 was amplified without its MDM2 binding domain (MBD) through PCR using 5′-GCGCGCGCGCGGTACCGCTCCCAGAATGCCAGAGGC-′3 and 5′-GCGCGCG-GATCCTCAGTCTGAGTCAGGCCCTTCTGTC-3′. This was subcloned into the KpnI and BamHI restriction enzyme sites in EGFP-PS (*32*).

Cell Lines and Transient Transfections

1471.1 murine adenocarcinoma cells (gift of G. Hager, NCI, NIH) were grown as monolayers in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin-glutamine (Invitrogen), and 0.1% gentamicin (Invitrogen). The cells were maintained in a 5% CO_2 incubator at 37ºC. 7.5×10^4 cells were seeded in 2-well live cell chambers (Nalgene Nunc, Rochester, NY). Transfections were carried out 24 hours after seeding using 1 pmol DNA and Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. Constructs were induced with 100 nM of the dex ligand, 24 hours after transfection for 1 hour (*30*).

Fluorescence Microscopy

Approximately 24 hours after transfection, protein localization was viewed by fluorescence microscopy. Prior to live-cell imaging, media in live cell chambers was replaced with phenol red-free DMEM (Invitrogen) containing 10% charcoal-stripped fetal bovine serum (CS-FBS, Invitrogen). Images were acquired as previously (*33*), using an Olympus IX71F fluorescence microscope (Scientific Instrument Company, Aurora, CO) with high-quality narrow band GFP filter (ex: HQ480/20 nm, em: HQ510/20 nm) from Chroma Technology (Brattleboro, VT) with a 40X PlanApo oil immersion objective (NA 1.00) on an F-View Monochrome CCD camera. The microscope stage was maintained at 37°C with an air stream incubator (Nevtek ASI 400, Burnsville, VA). All

experiments were repeated in triplicates (n=3) and 10 cells were analyzed for each time-point in each experiment.

Treatment with LMB and Nutlin-3

Cells were incubated with 10 nM leptomycin B (LMB, an inhibitor of nuclear export) 10 hours after transfection and 14 hours before ligand induction with dex followed by microscopy. Another population of transfected cells was incubated with 10 μM nutlin-3 one hour before ligand induction (Sigma, St. Louis, MO).

Results and Discussion

Our previous studies have shown that the localization of exogenous proteins can be controlled by fusing a protein switch (PS) containing a nuclear localization signal (NLS), a nuclear export signal (NLS), and a ligand binding domain (LBD) (*29, 30, 34, 35*). The fused protein is initially concentrated in the cytoplasm followed by localization to the nucleus upon ligand addition (Figure 6.2a). In this study, we aimed to rationally design a proteasomal PS, which is cytoplasmic in the absence of ligand and proteasomal in the presence of ligand. We took advantage of the p53/MDM2 ubiquitin proteasome system when designing the PS.

The tumor suppressor p53 was fused to EGFP-PS forming a construct whose localization is controlled by a ligand, dex. In the absence of ligand, the protein localized to the cytoplasm. But unlike EGFP-PS alone, EGFP-PS-p53 localized to the proteasome (which has a characteristic punctate dot formation (*36*)) upon ligand induction (Figure 6.2b). The proteasomal targeting occurred within 30 minutes after the addition of dex.

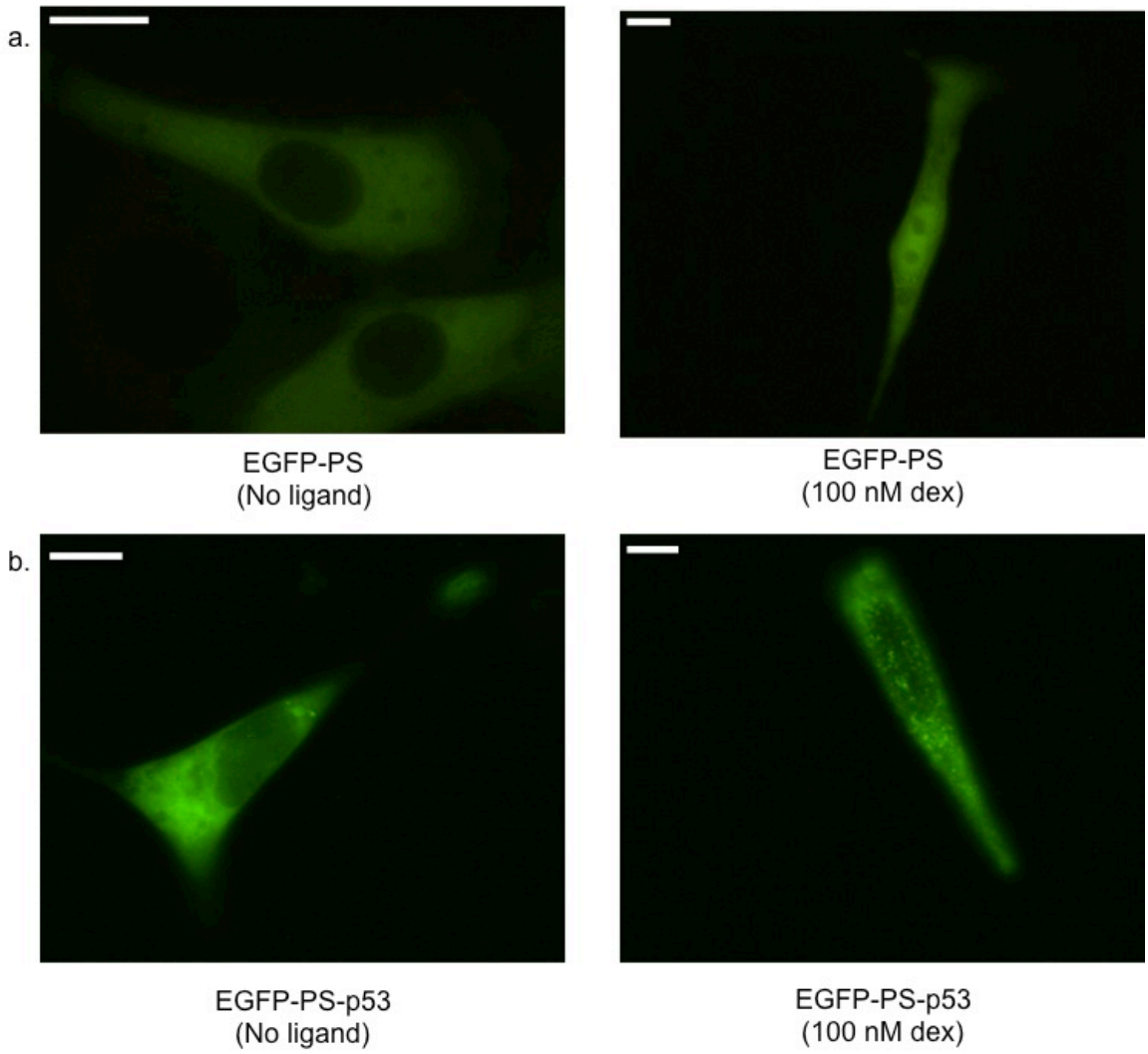


Figure 6.2. Change in protein localization on ligand induction. Change in localization of a) EGFP-PS and b) EGFP-PS-p53 are observed after induction with 100 nM dex for 1 hour. White scale bars are all 10 μm.

Cells were captured using live cell fluorescence microscopy every 5 minutes for 1 hour. A representative set of pictures over 30 minutes at 100 nM dex dose for EGFP-PS-p53 is shown in Figure 6.3. We speculate that fusing EGFP and PS to p53 makes it harder for p53 to form a tetramer. When the p53 is in the monomer or dimer form, it is easier for MDM2 to bind and export it from the nucleus (*37*). This might explain why EGFP-p53 was localized into the nucleus without proteasomal degradation (data not shown).

The proposed mechanism of our construct is still dependent on nuclear localization. We propose that EGFP-PS-p53 is localized to the nucleus upon ligand addition. However, the MDM2 protein binds to the construct causing its ubiquitination, nuclear export, and ultimately proteasomal targeting (Figure 6.4). To examine if this process is initiated because of the MDM2 pathway, we cloned a p53 deficient of the MDM2 binding domain (ΔMBD) to the EGFP-PS. This construct was initially concentrated in the cytoplasm and mainly localized to the nucleus upon addition of dex (Figure 6.5). To further explore the MDM2 pathway, cells were incubated with nutlin-3, which is a small molecule that interacts with MDM2 at the binding pocket of p53 preventing p53 binding (*25, 26*). Cells transfected with EGFP-PS-p53 and treated with both nutlin-3 and dex showed similar results to EGFP-PS-p53 ΔMBD (Figure 6.6).

To investigate the ability of MDM2 to export our construct from the nucleus to the cytoplasm, cells were incubated with leptomycin B (LMB). LMB is an inhibitor of CRM1 (chromosome region maintenance/exportin 1), which is a protein required for the nuclear export of proteins such as MDM2 and ubiquitinated p53 (*38, 39*). Cells transfected with EGFP-PS-p53 and treated with both LMB and dex showed nuclear

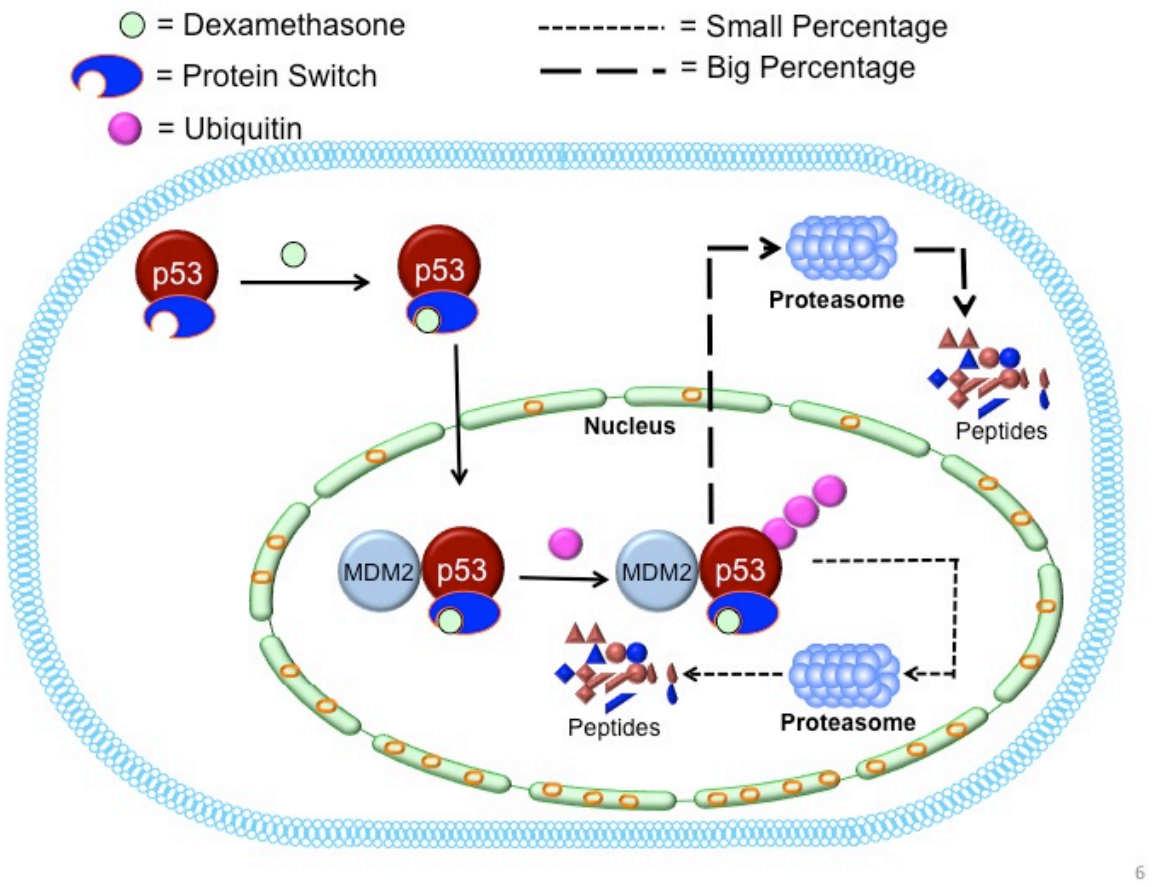


Figure 6.3. The proteasomal switch mechanism. p53-PS is expressed in the cytoplasm and is targeted to the nucleus upon ligand induction. MDM2 binds to p53 in the nucleus causing ubiquitination of p53. A small percentage of the ubiquitinated p53 is sent to nuclear proteasome for degradation. The majority of the ubiquitinated p53 is exported back to the cytoplasm and targeted to the cytoplasmic proteasome for degradation.

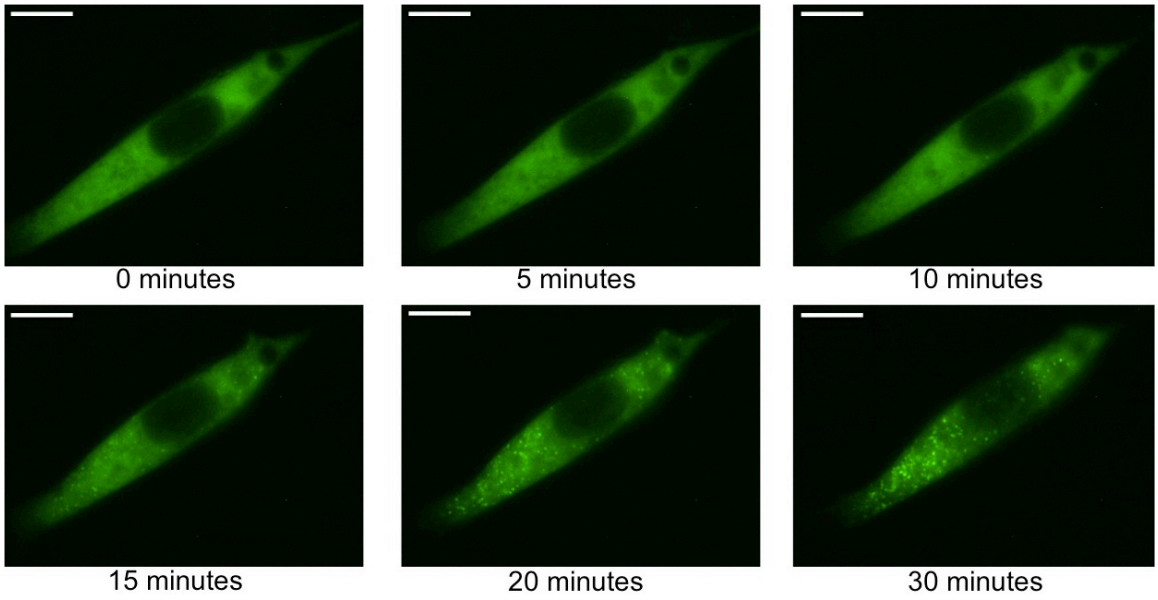


Figure 6.4. Representative time-dependent targeting for protein constructs. The figure shows representative time-dependent targeting for EGFP-PS-p53 with 100 nM dex. The movement of the protein could be observed from the cytoplasm to the proteasome over time upon ligand induction. White scale bars are all 10 μm.

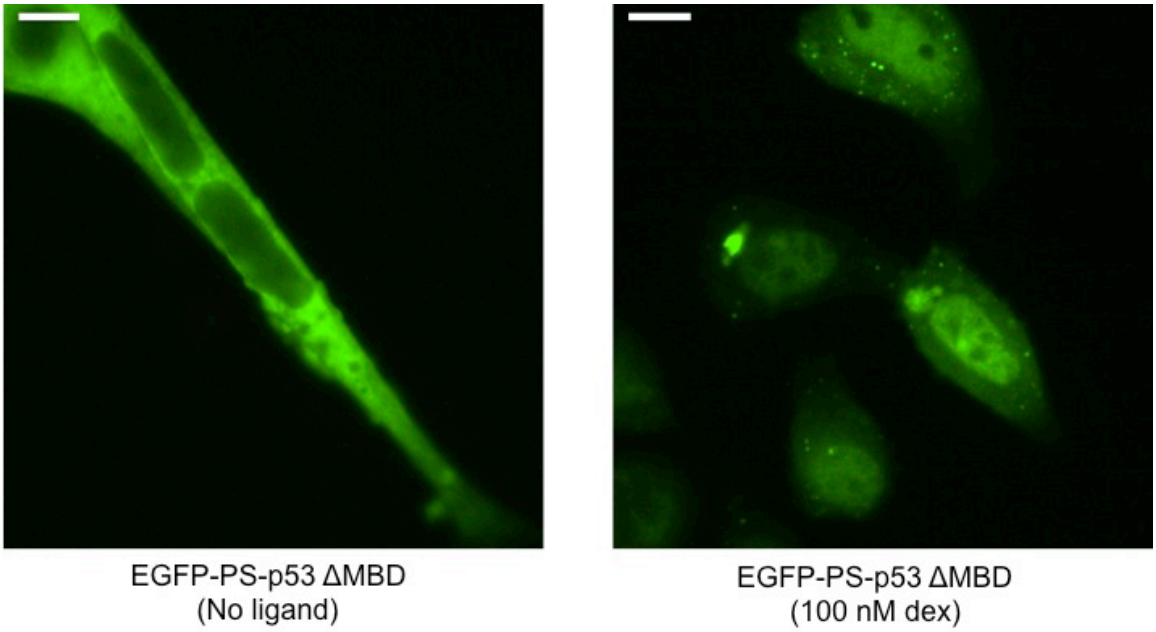


Figure 6.5. Change in EGFP-PS-p53 ΔMBD localization on ligand induction. Change in localization of protein is observed after induction with 100 nM dex for 1 hour. White scale bars are all 10 μm.

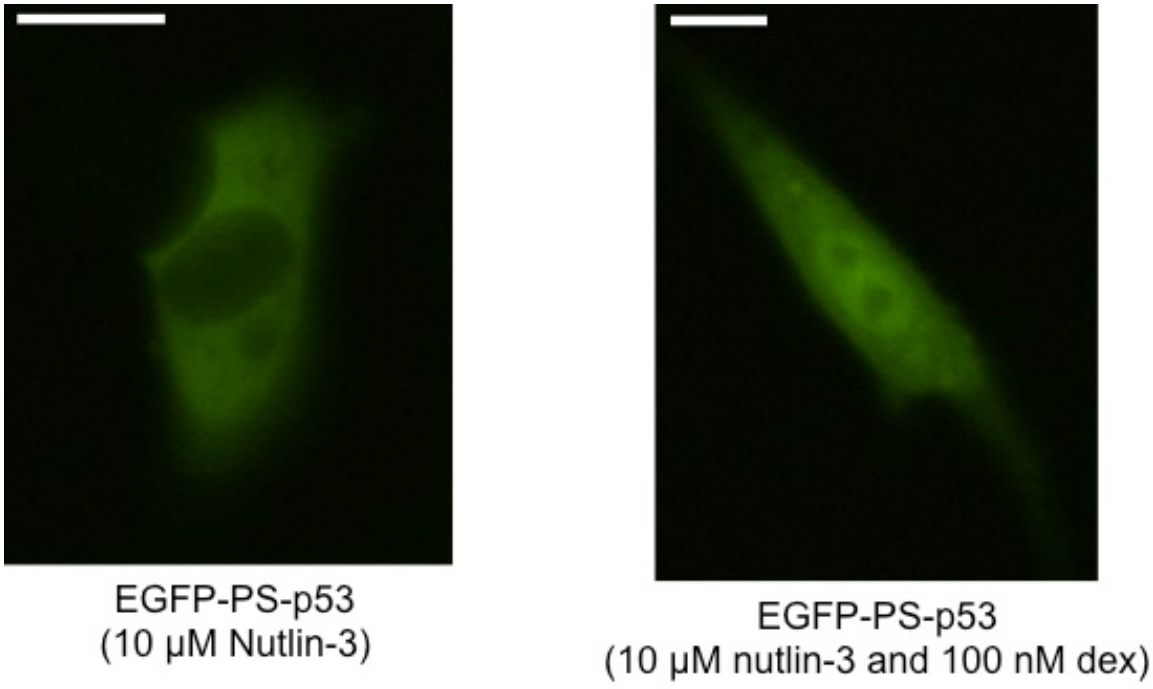


Figure 6.6. The effect of nutlin-3. Change in localization of protein is observed after induction with 100 nM dex for 1 hour preceded with 1 hour of incubation with 10 μM nutlin-3. White scale bars are all 10 μm.

localization of the constructs with some targeting to nuclear proteasome and no targeting to cytoplasmic proteasome (Figure 6.7). While LMB was able to prevent nuclear exclusion, it was not able to prevent targeting the limited nuclear proteasome (*17, 40*).

Like nature, the protein switch was designed to regulate cellular proteins by changing their location and hence their function (*41-43*). The protein switch has the advantage of being regulated by externally added ligand (*29, 30, 34*). The proteasomal protein switch can be engineered with a dimerization domain of a protein of interest, which will allow it to "capture" a cytoplasmic endogenous protein and send it for proteasomal degradation upon ligand induction. Since the ubiquitin ligase (E3) is what mediates substrate specificity (*2*), we envision the use of the constructed proteasomal protein switch as a therapeutic to target oncogenic proteins. Depleting cytoplasmic oncogenic proteins such as Bcr-Abl, survivin, p27, and Raf-1 (*33, 44-46*) would be a specific treatment for cancer cells. This might prove useful in cancer with elevated levels of MDM2 such as breast cancer, melanoma, esophageal cancer, leukemia, sarcoma, non-small cell lung cancer, and non-hodgkin's lymphoma (*18*). In addition, the EGFP-PS-p53 construct could also be used as a p53-MDM2 binding assay using fluorescence microscopy to screen for inhibitors that could disrupt the p53-MDM2 interaction. Nutlin-3, a MDM2 inhibitor was used to confirm the feasibility of the assay.

Conclusion

A proteasomal protein switch was constructed taking advantage of the degradation pathway of p53. A protein switch containing EGFP, a NLS, a NES, a GRLBD, and p53 was cloned. We provided indirect evidence that the expressed protein

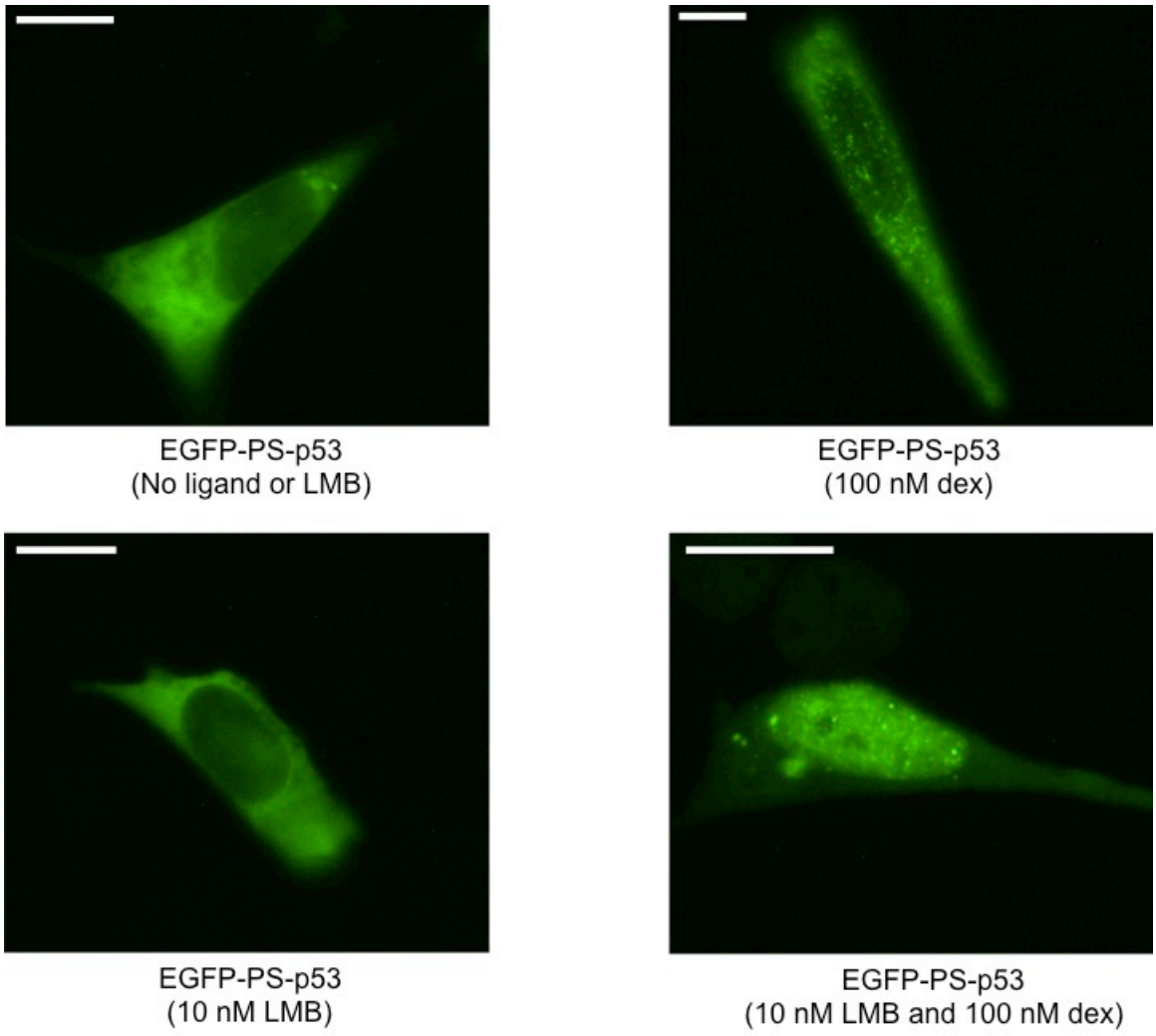


Figure 6.7. The effect of LMB. Change in localization of protein is observed after induction with 100 nM dex for 1 hour preceded with 14 hours of incubation with 10 nM LMB. White scale bars are all 10 μm.

is localized to the cytoplasm and is moved to the proteasome upon the addition of dex. The proteasomal targeting was achieved via binding to and nuclear exclusion via the ubiquitin ligase, MDM2. Preliminary studies shown here indicate that this mechanism is feasible by using LMB, nutlin-3, and p53 ΔMBD. Indirect immunefluorescence and western blotting will be performed to prove proteasomal targeting and protein degradation. Further studies are expected to investigate the application of this switch to capture cytoplasmic oncogenic proteins and send them for degradation upon ligand induction. The proteasomal switch could also be used as an alternative microscopy-based screening for inhibitors of the p53/MDM2 interaction.

References

1. Nalepa, G., and Wade Harper, J. (2003) Therapeutic anti-cancer targets upstream of the proteasome, *Cancer Treat. Rev. 29 Suppl 1*, 49-57.

2. Nalepa, G., Rolfe, M., and Harper, J. W. (2006) Drug discovery in the ubiquitin-proteasome system, *Nat. Rev. Drug Discov. 5*, 596-613.

3. Dahlmann, B. (2007) Role of proteasomes in disease, *BMC Biochem. 8 Suppl 1*, S3.

4. Vucic, D., Dixit, V. M., and Wertz, I. E. (2011) Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death, *Nat. Rev. Mol. cell Biol. 12*, 439-452.

5. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53, *Nature 387*, 296-299.

6. Chan, W. M., Mak, M. C., Fung, T. K., Lau, A., Siu, W. Y., and Poon, R. Y. (2006) Ubiquitination of p53 at multiple sites in the DNA-binding domain, *Mol. Cancer Res. 4*, 15-25.

7. Vousden, K. H., and Lane, D. P. (2007) p53 in health and disease, *Nat. Rev. Mol. Cell Biol. 8*, 275-283.

8. Vousden, K. H., and Lu, X. (2002) Live or let die: the cell's response to p53, *Nat. Rev. Cancer 2*, 594-604.

9. Espinosa, J. M. (2008) Mechanisms of regulatory diversity within the p53 transcriptional network, *Oncogene 27*, 4013-4023.

10. Murray-Zmijewski, F., Slee, E. A., and Lu, X. (2008) A complex barcode underlies the heterogeneous response of p53 to stress, *Nat. Rev. Mol. Cell Biol. 9*, 702-712.

11. Riley, T., Sontag, E., Chen, P., and Levine, A. (2008) Transcriptional control of human p53-regulated genes, *Nat. Rev. Mol. Cell Biol. 9*, 402-412.

12. Fakharzadeh, S. S., Trusko, S. P., and George, D. L. (1991) Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line, *EMBO J. 10*, 1565-1569.

13. Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. (1993) The p53-mdm-2 autoregulatory feedback loop, *Genes Dev. 7*, 1126-1132.

14. Bond, G. L., Hu, W., and Levine, A. J. (2005) MDM2 is a central node in the p53 pathway: 12 years and counting, *Curr. Cancer Drug Targets 5*, 3-8.

15. Piette, J., Neel, H., and Marechal, V. (1997) Mdm2: keeping p53 under control, *Oncogene 15*, 1001-1010.

16. Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998) Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein, *EMBO J. 17*, 554-564.

17. Stommel, J. M., and Wahl, G. M. (2004) Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation, *EMBO J. 23*, 1547-1556.

18. Momand, J., Jung, D., Wilczynski, S., and Niland, J. (1998) The MDM2 gene amplification database, *Nucleic Acids Res. 26*, 3453-3459.

19. Onel, K., and Cordon-Cardo, C. (2004) MDM2 and prognosis, *Mol. Cancer Res. 2*, 1-8.

20. Wasylyk, C., Salvi, R., Argentini, M., Dureuil, C., Delumeau, I., Abecassis, J., Debussche, L., and Wasylyk, B. (1999) p53 mediated death of cells overexpressing MDM2 by an inhibitor of MDM2 interaction with p53, *Oncogene 18*, 1921-1934.

21. Chene, P., Fuchs, J., Bohn, J., Garcia-Echeverria, C., Furet, P., and Fabbro, D. (2000) A small synthetic peptide, which inhibits the p53-hdm2 interaction, stimulates the p53 pathway in tumour cell lines, *J. Mol. Biol. 299*, 245-253.

22. Wang, H., Nan, L., Yu, D., Lindsey, J. R., Agrawal, S., and Zhang, R. (2002) Anti-tumor efficacy of a novel antisense anti-MDM2 mixed-backbone oligonucleotide in human colon cancer models: p53-dependent and p53-independent mechanisms, *Mol. Med. 8*, 185-199.

23. Kussie, P. H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A. J., and Pavletich, N. P. (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain, *Science 274*, 948-953.

24. Patel, S., and Player, M. R. (2008) Small-molecule inhibitors of the p53-HDM2 interaction for the treatment of cancer, *Expert. Opin. Investig. Drugs 17*, 1865-1882.

25. Klein, C., and Vassilev, L. T. (2004) Targeting the p53-MDM2 interaction to treat cancer, *Br. J. Cancer 91*, 1415-1419.

26. Vassilev, L. T. (2004) Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics, *Cell Cycle 3*, 419-421.

27. Ylikomi, T., Bocquel, M. T., Berry, M., Gronemeyer, H., and Chambon, P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors, *EMBO J. 11*, 3681-3694.

28. Vegeto, E., Allan, G. F., Schrader, W. T., Tsai, M. J., McDonnell, D. P., and O'Malley, B. W. (1992) The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor, *Cell 69*, 703-713.

29. Kanwal, C., Mu, S., Kern, S. E., and Lim, C. S. (2004) Bidirectional on/off switch for controlled targeting of proteins to subcellular compartments, *J. Control. Release 98*, 379-393.

30. Kakar, M., Davis, J. R., Kern, S. E., and Lim, C. S. (2007) Optimizing the protein switch: altering nuclear import and export signals, and ligand binding domain, *J. Control. Release 120*, 220-232.

31. Wan, Y., Coxe, K. K., Thackray, V. G., Housley, P. R., and Nordeen, S. K. (2001) Separable features of the ligand-binding domain determine the differential subcellular localization and ligand-binding specificity of glucocorticoid receptor and progesterone receptor, *Mol. Endocrinol. 15*, 17-31.

32. Htun, H., Barsony, J., Renyi, I., Gould, D. L., and Hager, G. L. (1996) Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera, *PNAS USA 93*, 4845-4850.

33. Dixon, A. S., Kakar, M., Schneider, K. M., Constance, J. E., Paullin, B. C., and Lim, C. S. (2009) Controlling subcellular localization to alter function: Sending oncogenic Bcr-Abl to the nucleus causes apoptosis, *J. Control. Release 140*, 245-249.

34. Davis, J. R., Kakar, M., and Lim, C. S. (2007) Controlling protein compartmentalization to overcome disease, *Pharm. Res. 24*, 17-27.

35. Kakar, M., Cadwallader, A. B., Davis, J. R., and Lim, C. S. (2007) Signal sequences for targeting of gene therapy products to subcellular compartments: the role of CRM1 in nucleocytoplasmic shuttling of the protein switch, *Pharm. Res. 24*, 2146-2155.

36. Chan, N. C., Salazar, A. M., Pham, A. H., Sweredoski, M. J., Kolawa, N. J., Graham, R. L., Hess, S., and Chan, D. C. (2011) Broad activation of the ubiquitin-

proteasome system by Parkin is critical for mitophagy, *Hum. Mol. Gen. 20*, 1726-1737.

37. Hjerpe, R., Aillet, F., Lopitz-Otsoa, F., Lang, V., Torres-Ramos, M., Farras, R., Hay, R. T., and Rodriguez, M. S. (2010) Oligomerization conditions Mdm2-mediated efficient p53 polyubiquitylation but not its proteasomal degradation, *Intl J. Biochem. Cell Biol. 42*, 725-735.

38. Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998) Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1, *Exp. Cell Res. 242*, 540-547.

39. Lohrum, M. A., Woods, D. B., Ludwig, R. L., Balint, E., and Vousden, K. H. (2001) C-terminal ubiquitination of p53 contributes to nuclear export, *Mol. Cellular Biol. 21*, 8521-8532.

40. Joseph, T. W., Zaika, A., and Moll, U. M. (2003) Nuclear and cytoplasmic degradation of endogenous p53 and HDM2 occurs during down-regulation of the p53 response after multiple types of DNA damage, *FASEB J. 17*, 1622-1630.

41. Lowe, S. W., and Sherr, C. J. (2003) Tumor suppression by Ink4a-Arf: progress and puzzles, *Curr. Opin, Genet, Dev, 13*, 77-83.

42. Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops, *Oncogene 24*, 2899-2908.

43. Honda, R., and Yasuda, H. (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53, *EMBO J. 18*, 22-27.

44. Wen, S., So, Y., Singh, K., Slingerland, J. M., Resnick, M. B., Zhang, S., Ruiz, V., and Moss, S. F. (2011) Promotion of cytoplasmic mislocalization of p27 by Helicobacter pylori in gastric cancer, *Oncogene [Epub ahead of print] 21841827.*

45. Wood, K. W., Qi, H., D'Arcangelo, G., Armstrong, R. C., Roberts, T. M., and Halegoua, S. (1993) The cytoplasmic raf oncogene induces a neuronal phenotype in PC12 cells: a potential role for cellular raf kinases in neuronal growth factor signal transduction, *PNAS USA 90*, 5016-5020.

46. Rexhepaj, E., Jirstrom, K., O'Connor, D. P., O'Brien, S. L., Landberg, G., Duffy, M. J., Brennan, D. J., and Gallagher, W. M. (2010) Validation of cytoplasmic-to-nuclear ratio of survivin as an indicator of improved prognosis in breast cancer, *BMC Cancer 10*, 639.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

Conclusions

This thesis described the delivery of therapeutics (small molecules or biologics) to achieve a certain effect at a specific subcellular compartment (*1*). The main theme of this research is to use subcellular targeting to elicit or enhance a therapeutic effect, which leads to the hypothesis that *targeting specific organelles in a cell will lead to better drug delivery and improvements in disease therapy*. The delivery of the adiponectin protein was optimized to affect the oxidative stress mechanism in the mitochondria. Vitamin E was also conjugated to a cation to enhance its mitochondrial targeting where it acts as an antioxidant. Mitochondrial targeting was further investigated to deliver proteins such as p53 to induce apoptosis in cancer cells. In addition, proteasomal targeting was explored to create a ligand inducible protein switch to the proteasome.

Adiponectin Released from the Triblock Copolymer Maintained Its Antioxidant Effect

The concept of using a biodegradable polymer for constant release of a therapeutic protein offers novel treatment therapies for cardiovascular disease and diabetes (*2*). The triblock copolymer used contains a hydrophilic polyethylene glycol

(PEG) block and two hydrophobic poly(lactide-co-glycolide) (PLGA) blocks (*3, 4*). The copolymer is arranged as PLGA-PEG-PLGA, which allows the delivery of insoluble or unstable therapeutics. The polymer is in the liquid form at low temperature but becomes a hydrogel at body temperature. The goal of using the biodegradable polymer is to maintain a controlled delivery of a therapeutic at a specific site such as the heart (*5, 6*). The globular form of adiponectin (gAdp) was solubilized in the polymer gel and was completely released over 27 days without an initial burst. The protein was not degraded or aggregated after being released from the polymer gel. The released gAdp from the triblock copolymer maintained its activity in reducing reactive oxygen species (ROS) in endothelial cells via cAMP/PKA pathway. The ability of reducing oxidative stress was measured in hyperglycemic cells, which overproduce ROS via the mitochondrial electron transport chain (*7*). This indicates that injectable gels containing gAdp could be used as a formulation to reduce oxidative stress if injected locally to the heart. The hydrogel depot containing gAdp could also be injected subcutaneously to compensate the reduction of adiponectin in the plasma of diabetic patients.

A Faster Synthesis of Mitochondrially Targeted Vitamin E for Reversal of Oxidative Stress

A faster and a more efficient method to conjugate vitamin E to triphenylphosphonium (TPP) was designed. The conjugation was carried out using a lysine linker and solid phase synthesis. Targeting the mitochondria for reducing oxidative stress has been under investigation because of the mitochondria's role in producing and metabolizing ROS. Targeting antioxidants to the mitochondria can be amplified by

conjugating a cation taking advantage of the mitochondrial transmembrane potential (*1, 8-10*). Conjugating antioxidants to TPP has been previously conducted but with a more complicated scheme (*11*). Solid phase synthesis does not require isolation of synthetic intermediates. In addition, all reagents and byproducts are easily washed out after each step. A lysine linker with two protecting groups enabled the conjugation of vitamin E and TPP. The resultant MitoE was able to localize and concentrate into the myocardial mitochondria of treated mice. MitoE also showed better reduction of oxidative stress in hyperglycemic endothelial cells than vitamin E alone. This method of conjugating TPP to vitamin E should be further investigated with other antioxidants.

Mitochondrial p53 Activity Depends on the Mitochondrial Signal Used

Mitochondrial targeting of p53 triggers a rapid apoptotic response by releasing cytochrome c (*12, 13*). p53 induces the oligomerization of the pro-apoptotic Bak and Bax (*14-16*) by directly binding to them (*17*) or binding to the anti-apoptotic Bcl-XL (*18*). Since subcellular targeting is crucial for protein activity, we explored targeting mitochondrial subcompartments. p53 was targeted to the outer surface of the mitochondria via fusing to the mitochondrial signal (MTS) from Bcl-XL (XL) (*19-22*). It was also inserted into the outer membrane and the inner membrane by fusing the MTSs from TOM20 (TOM) (*19*) and cytochrome c oxidase (CCO) (*23, 24*), respectively. The mitochondrial matrix was also targeted by fusing p53 to the MTS from ornithine transcarbamylase (OTC) (*25, 26*). In addition, EGFP was also fused to all MTSs to examine their innate toxicity. EGFP fused to XL showed minimum toxicity, while EGFP

fused to the other three MTSs initiated apoptosis. This concludes that fusing EGFP to TOM, CCO, or OTC has a toxic effect on the mitochondria. From our microscopy and luciferase assay data, it was discovered that efficiency in targeting the mitochondria depends on the strength of the MTS especially when fusing a protein that contains nuclear localization signals (NLSs) such as p53 (*27*). XL and TOM showed maximum strength in targeting the mitochondria followed by OTC and then CCO being the weakest signal. Because of their weak signal strength, OTC-p53 and CCO-p53 showed nuclear activity in addition to their mitochondrial activity. On the other hand, TOM-p53 and p53-XL had no nuclear activity. Based on our apoptotic assays (caspase-9, TUNEL, Annexin-V, and 7AAD) and rescue experiments (Bcl-XL, pifithrin-α, and pifithrin-μ), TOM-p53 is speculated to initiate apoptosis through direct binding to Bak. The p53-XL construct was the most promising and specific to p53/Bcl-XL mitochondrial pathway. Further experiments will be carried out to explore the p53 binding to Bcl-XL using smaller domains of p53 and introducing p53 mutations to increase the binding.

Proteasomal Localization Controllable Construct

The concept of using a protein switch for changing intracellular localization of proteins from cytoplasm to proteasome offers novel treatment therapies for diseases caused by cytoplasmic oncogenic/aberrant proteins. A proteasomal protein switch was constructed containing EGFP, a NLS, a nuclear export signal (NES), a ligand binding domain from the glucocorticoid receptors (GRLBD), and p53. The protein switch without p53 localizes to the cytoplasm and then is targeted to the nucleus upon the addition of ligand (*28-30*). However, the expressed protein switch, containing p53, localized

predominately in the cytoplasm in the absence of ligand and translocated to the proteasome on ligand induction. Our initial studies (using leptomycin B, nutlin-3, and p53 ΔMDM2 binding domain), while not complete, indicate indirect evidence that the proteasomal targeting was achieved via binding to and nuclear exclusion by MDM2. We speculate that p53 cloned into our protein switch binds to MDM2 in the nucleus upon ligand addition. MDM2, an ubiquitin ligase (E3), helps the transfer of ubiquitin from E2 to the lysine residues on the carboxy terminus of p53 (*31-33*). Subsequently, the proteasome recognizes the poly-ubiquitinated protein for degradation.

Future Work

The long term objective of this research is to exploit different mechanisms of targeting the various cellular compartments to enhance drug delivery and disease therapy. The tested hydrogel formulation containing adiponectin should be further examined in vivo by either subcutaneous injection or local delivery to the heart. The chemistry used to conjugate TPP to vitamin E should be taken advantage of to conjugate other antioxidants and test their mitochondrial activity. In addition, the mitochondrial p53 will be further explored by identifying the p53 domain that binds best to Bcl-XL. The p53 binding to Bcl-XL will also be enhanced by introducing point mutations in p53 to increase the hydrophobic and electrostatic interactions. Moreover, p53 will be modified to bind to Bak on the outer surface of the mitochondria. The transcriptional activity of p53 will also be explored by controlling nuclear localization and increasing tetramerization and nuclear accumulation. Finally, the proteasomal protein switch will be tested in leukemia cell lines to deplete the cytoplasmic Bcr-Abl, oncogenic protein.

Antioxidant Potential of Controlled Released Adiponectin *In Vivo*

The ability of adiponectin to reduce oxidative stress in type 2 diabetes (db/db mice) using a triblock copolymer formulation should be assessed. Other studies have shown promising results in vivo using other proteins such as interleukin-2 (IL-2), porcine growth hormone (pGH), and PEGylated camptothecin (*34-36*). IL-2 loaded hydrogel showed an enhanced anti-tumor effect when injected subcutaneously to H22 tumor bearing mice (*34*). The formulation containing pGH and PEGylated camptothecin were also delivered subcutaneously in rabbits and S-180 sarcoma-bearing Kunming mice, respectively (*35, 36*). In addition, type 2 diabetic rats were also used as a model for subcutaneous delivery of incretin hormone glucagon-like peptide-1 (GLP-1) and insulin in a hydrogel formulation to increase plasma insulin levels (*37, 38*).

After db/db mice are subcutaneously injected with the hydrogel formulation containing gAdp, blood plasma will be collected over a period of 10 days to note elevation of adiponectin in the blood stream. Echocardiogram will also be obtained before and after treatment. After the 10 days, mice will be sacrificed and their hearts will be harvested. Myocardial mitochondria will be isolated to determine rates of mitochondrial oxygen consumption, ATP generation (to analyze mitochondrial uncoupling), and mitochondrial hydrogen peroxide generation.

Conjugation of Antioxidants to TPP Using Solid Phase Synthesis

TPP conjugation via solid phase synthesis should be explored with other antioxidants such as co-enzyme Q and quercetin. Mitochondrially targeted co-enzyme Q (MitoQ) shows promising results in preventing loss of spatial memory retention and early neuropathology in transgenic mice with Alzheimer's disease by reducing ROS (*39*). In addition, quercetin conjugated to TPP accumulates in the mitochondria and inhibits mitochondrial ATPase activity (*40*). The TPP conjugation to the different antioxidants should be investigated using different amino acid linkers (such as lysine versus glycine). The effect of the length, hydrophobicity, and charge of the amino acid on the activity of the final product should be explored. The activity of the conjugated amino acid will be tested first *in vitro* in endothelial cells measuring their ability to reduce ROS as was performed in our previous study (*2*).

The conjugated antioxidants should be further investigated *in vivo*. Diabetic mice will be given the antioxidant in their drinking water for two week. The mice will be sacrificed and their hearts harvested. After the isolation of mitochondria, the amount of Mitochondrially targeted antioxidants will be measured with the rates of mitochondrial oxygen consumption, ATP generation, and mitochondrial hydrogen peroxide generation. If the triblock copolymer mentioned above is proven successful in vivo with adiponectin, it should be used to deliver the newly TPP conjugated antioxidants.

Targeting Small Domains of p53 to Mitochondrial Bcl-XL

This study shows that fusing p53 to XL expressed optimal mitochondrial targeting, highest specificity to the p53/Bcl-XL apoptotic pathway, and lowest MTS toxicity when fused to EGFP. Truncated domains of p53 will be fused to XL to better understand p53/Bcl-XL binding and to create the smallest mitochondrial p53 version that will maintain its activity. There are conflicting reports on how p53 binds to Bcl-XL. There are multiple studies claiming that p53 binds to Bcl-XL through its DNA binding domain (DBD, amino acids 102-292) (Figure 1.5) (*26, 41, 42*). Other reports affirm that the amino terminus domain (NTD, amino acids 1-101) is sufficient in binding to Bcl-XL (*15, 43, 44*). The NTD contains the transactivation domain (TAD, amino acids 1-62) and the proline rich domain (PRD, amino acids 63-101). There is also a study that shows efficient induction of intrinsic apoptosis when sending p53 ΔC-terminus (amino acids 1-305) to the mitochondria. Another study explains the effective binding of MDM2 binding domain (MBD, amino acids 15-29) of p53 to Bcl-XL (*45*).

Future studies will fuse different domains of p53 to XL and examine their apoptotic activities. The following seven domains of p53 fused to XL will be compared to the wild type p53 (amino acids 1-393) fused to XL: DBD (amino acids 102-292), NTD (amino acids 1-101), MBD (amino acids 15-29), PRD (amino acids 63-101), PRD-DBD (amino acids 63-292), p53 ΔC-terminus (amino acids1-305), and p53 ΔMBD (amino acids 63-393). The following apoptotic assays will be performed: capase-9, TUNEL, Annexin-V, and 7-AAD. Rescue experiments using pifithrin-μ and over-expression of Bcl-XL will be carried out to confirm p53/Bcl-XL pathway as in Chapter 5. Mammalian

two-hybrid assay will also be performed to verify interaction of p53 domains with Bcl-XL. The construct with the highest apoptotic potential (similar to wild type p53) will give us a better insight on which domain of p53 is efficient to induce apoptosis by binding to Bcl-XL. The minimal domains necessary to induce apoptosis at the mitochondria will decrease the overall size of the final plasmid allowing better transfection efficiency in cell lines (*46*). This study will be performed on T47D, and MCF-7 cell lines as in Chapter 5. This work is currently in progress by Karina Matissek in the Lim lab.

Creating "Super" Mitochondrial p53

To further investigate the interaction of p53 with Bcl-XL, certain mutations will be introduced to p53-XL. The point mutations will be introduced to the amino acids that have been reported to bind to Bcl-XL. Mutations will be introduced to the DBD in the following amino acids: G117, S121, V173, C176, H178, C182, C238, N239, S241, M243, R248, C277, G279, and R280 (*18, 42, 47*). We will explore the hydrophobic and electrostatic interactions among residues. We will start with N239 and C176 since they are involved in binding to both Bcl-XL and Bcl2 (*42*). The proposed mutations for these two amino acids are N239Q, N239Y, C176V, C176M, C176F, and C176I. Since asparagine (N) forms a hydrogen bond with Bcl-XL, introducing glutamine (Q) or tyrosine (Y) might show a better interaction. The longer chain on Q will shorten the distance for better hydrogen bonding. On the other hand, the hydroxyl group on Y might give a better alternative for hydrogen bonding instead of the amino group on N. In addition, the hydrophobic interaction from cysteine (C) will be further explored by replacing it with more hydrophobic amino acids: valine (V), methionine (M),

phenylalanine (F), or isoleucine (I). Moreover, mutations to the MBD will also be introduced to p53-XL. The following amino acids have been reported to bind to Bcl-XL: Q16, F19, S20, L22, W23, L25, L26, and P27 (*45*). We will start with W23 and L26. The proposed mutations for these two amino acids are W23F, L26F, and L26I. The hydrophobic interaction from tryptophan (W) and leucine (L) will be enhanced by replacing them with more hydrophobic amino acids: F and I. All constructs will be tested on the following apoptotic assays: capase-9, TUNEL, Annexin-V, and 7-AAD. Mammalian two-hybrid assay will also be performed to verify interaction of p53 domains with Bcl-XL. This work is currently in progress by Karina Matissek in the Lim lab.

Enhanced Dimerization of p53 to Target Mitochondrial Bak

In addition to binding p53 to Bcl-XL, targeting Bak in the mitochondria will be explored. p53 can bind directly to Bak to induce its oligomerization causing mitochondrial outer membrane permeabilization (*16, 17*). Bak is a member of the Bcl2 family protein and is found on the outer surface of the mitochondria (*48*). We plan on fusing the MTS from Bak or Bax to p53 to ensure its Bak targeting. It is important to note that the tetramerization domain (TD) is crucial for p53 binding to Bak (*17*). Any mutation or deletion of TD weakens the ability of p53 to interact with Bak. p53 has to either be in the dimer or tetramer form to ensure binding (*17*). Therefore we plan to only use full length p53 in this project. We will also investigate increasing the tetramerization/dimerization of p53 to enhance binding to Bak. This will be performed by replacing the TD from p53 with a coiled coil (CC) from Bcr protein (*49*) forming p53-

CC. More information on using CC will be discussed in the following section. Our constructs (p53 and p53-CC) fused to the MTSs from Bak or Bax will be tested in apoptotic assays as described above. The p53 and p53-CC interaction with Bak will be studied using the mammalian two-hybrid assay. We expect to see an increase in apoptotic activity with p53-CC-Bak. This work is currently in progress by both Abood Okal and Karina Matissek in the Lim lab.

Substituting Tetramerization Domain of p53 with Bcr Coiled Coil

A major barrier to using p53 for gene therapy stems from a "dominant negative" effect in certain cancers. In these cancers, p53 is mutated or mislocalized. Any exogenous wild type p53 forms a dimer/tetramer with the endogenous mutated p53, causing the inactivation of the added p53. To circumvent this problem, the TD of p53 will be replaced by the coiled-coil from Bcr. This p53-CC construct will only dimerize/tetramerize with itself, and not to the mutated p53 in cancer cells. As a result, there will be no dominant negative effect. The CC behaves similarly to TD in forming an antiparallel dimer of dimers (*49*). In addition, the removal of the carboxy terminus (Δ amino acids 323-293) will also eliminate the lysine residues that are ubiquitinated by MDM2. We expect the p53-CC to show elevation in transcriptional activity due to the stronger binding motif and the prevention of proteasomal degradation. The p53-CC should have a better impact on cells containing mutant p53 because of the inability to form hetero-tetramers. Our preliminary data using a transcription activity assay (Dual-Glo luciferase assay) suggests elevation in transcription activity of p53-CC compared to

wild type p53 (data not shown). The following apoptotic assays will be performed: capase-3/7, TUNEL, Annexin-V, and 7-AAD. Rescue experiments using pifithrin-α will be carried out to confirm DNA binding. Cell cycle arrest will also be assayed using DyeCycle^{TM} Ruby stain. The gene expression profile of p53-CC will also be investigated using a Signaling Pathway PCR Array. This work is currently in progress by Abood Okal in the Lim lab.

Fusing 4 NLSs to p53

Increased nuclear accumulation of exogenous p53 will be explored to increase its transcriptional activity. The accumulation of p53 in the nucleus is necessary to bind to DNA (*50, 51*). Proteins containing NLSs are recognized by the nuclear import receptors and are translocated to the nucleus. p53 already contains a medium strength NLS (amino acids 305-322) and two weak NLSs (amino acids 369-375 and 379-384). Mutating the medium strength NLS is sufficient to decrease nuclear targeting dramatically (*27*). We hypothesize that fusing a stronger NLS to p53 will increase its nuclear accumulation and hence its transcription activity. It might also decrease proteasomal degradation since having a stronger NLS might prevent MDM2 from dragging p53 out of the nucleus. Our preliminary data using a transcription activity assay (Dual-Glo luciferase assay) suggests that fusing one NLS from MycA8 protein to p53 increases its transcriptional activity compared to wild type p53 (data not shown). Our lab has also proved that fusing 4 NLSs from SV40 Large T-antigen to Bcr-Abl was able to translocate the protein into the nucleus better than 1 NLS (*52*). Therefore, we will use the same approach by fusing 4 NLSs from SV40 to p53. Moreover, multiple NLSs will also be fused to p53-CC to

increase its nuclear accumulation. Similar apoptotic and cell cycle arrest assays will be performed as described above. This work is currently in progress by Abood Okal in the Lim lab.

Controlled Nuclear Targeting of p53-CC

A p53 nuclear protein switch will be developed to control the localization and hence the activity of p53. When p53 was fused to our nuclear protein switch, it was targeted to the proteasome instead and was used as a proteasomal protein switch. To restore the nuclear p53 protein switch, the p53 proteasomal degradation will be blocked by removing the carboxy terminus, where the ubiquitinated lysine residues reside, and replacing it with a CC from Bcr as discussed above. The new construct (EGFP-PS-p53-CC) will be expressed in the cytoplasm. However we expect that it will localize to the nucleus upon ligand induction. The transcriptional activity of p53-CC should be activated upon the addition of ligand. The localization of the construct will be monitored using fluorescence microscopy. The apoptotic potential of the protein switch will be tested before and after ligand. The transcriptional activity will also be observed using a Dual-Glo luciferase assay. This work is currently in progress by Abood Okal in the Lim lab.

Application of the Protein Switch: Depleting Cytoplasmic Bcr-Abl in Chronic Myelogenous Leukemia

Bcr-Abl is an oncoprotein that is found in the cytoplasm of Chronic Myelogenous Leukemia (CML) (*53, 54*). Bcr-Abl interacts with multiple signal transduction pathways transmitting anti-apoptotic signals (*55*). Small molecules such as imatinib targeting Bcr-

Abl are usually the treatment of choice for CML (*56-58*). However some patients have developed resistance to imatinib due to mutations in the kinase domain of Bcr-Abl (*59-61*). Our lab has shown that nuclear p53 has apoptotic potential (*52*). In addition, the Lim lab has developed mutations in the CC domain of Bcr to enhance binding to Bcr-Abl (*62*). The CC domain with certain mutations was efficient in binding to Bcr-Abl and disrupting some of its activity (*62*). We plan to use the third generation of CC mutations (CC3) as a binding domain for Bcr-Abl. The mutations introduced increased hetero-oligomerization with Bcr-Abl and decreases homo-oligomerization of CC3. CC3 will be fused to our proteasomal protein switch (EGFP-PS-p53). We expect the construct to localize to the cytoplasm of k562 cells (CML cells) allowing oligomerization with endogenous Bcr-Abl. Upon ligand induction, we predict the localization of our protein switch along with Bcr-Abl to the proteasome causing its degradation. The feasibility of dragging Bcr-Abl to the proteasome will be confirmed using fluorescence microscopy and indirect immunofluorescence. Apoptotic assays will also be performed to investigate its apoptotic potential. This work is currently in progress by James R. Davis in the Lim lab.

Validating Our Therapy *In Vivo* Using an Adenovirus Vector

The optimized p53 plasmids (targeting the mitochondria and the nucleus) will be constructed into an adenoviral vector using Adeno-X Expression System 2 (Clontech). Adenovirus is the most efficient in terms of delivering their genetic cargo to the nucleus (*63-67*). Adenovirus containing p53 has been widely used in gene therapy clinical trials

(*73*). In addition, the immunogenicity of adenovirus has improved recently enhancing its prospects for long-term gene transfer in a wide range of different tissues (*68*). Although not without issues, adenovirus will be used in our lab's future studies as a means to validate our model as a high efficiency vector. The cancer specific surviving promoter (*69*) will also be used in the viral vector delivery system since we will be using breast cancer animal model. The therapeutic effect of nuclear and mitochondrial p53 and p53-CC will be tested in a human breast cancer xenograft model using female athymic nude mice (Balb/c nude; immunocompromised) subjected to right dorsa subcutaneous injection of MCF-7 cells (*70*). The adenoviral vector will be injected into the tumor. Tumor elimination or size reduction will be measured as an indicator of therapeutic efficacy. Toxicity/immunogenicity of adenoviral constructs will be tested as well. This work will be carried out by Karina Matissek, Abood Okal, and Shams Reaz in the Lim lab.

This dissertation and future work will offer a better understanding on subcellular delivery. The aim is to apply subcellular targeting to elicit or enhance therapeutic effect. By focusing on the targeting of subcellular organelles, we are taking therapeutic delivery to the next level. Controlled compartmentalization of proteins is already used by cells as a mechanism to regulate protein activity. This thesis and future work exploit these cellular mechanisms to alter the way gene therapy is approached, and could be applied to many other diseases besides the breast cancer and CML systems discussed here. The concept of controlled compartmentalization is particularly useful for gene therapy with p53, which is apoptotic in the nucleus and the mitochondria. It also represents a new era in drug design for therapy for cancers, where subcellular location alters function.

References

1. Mossalam, M., Dixon, A. S., and Lim, C. S. (2010) Controlling subcellular delivery to optimize therapeutic effect, *Ther. Deliv. 1*, 169-193.

2. Mossalam, M., Jeong, J. H., Abel, E. D., Kim, S. W., and Kim, Y. H. (2008) Reversal of oxidative stress in endothelial cells by controlled release of adiponectin, *J. Control. Release 130*, 234-237.

3. Kissel, T., Li, Y., Gorich, S., Volland, C., and Koneberg, R. (1996) Parenteral protein delivery systems using biodegradable ABA-block-copolymers, *J. Control. Release 39*, 315-326.

4. Duvvuri, S., Janoria, K. G., and Mitra, A. K. (2005) Development of a novel formulation containing poly(d,l-lactide-co-glycolide) microspheres dispersed in PLGA-PEG-PLGA gel for sustained delivery of ganciclovir, *J. Control. Release 108*, 282-293.

5. Chen, S., Pieper, R., Webster, D. C., and Singh, J. (2005) Triblock copolymers: synthesis, characterization, and delivery of a model protein, *Int. J. Pharm. 288*, 207-218.

6. Laurent, A., Nicco, C., Chereau, C., Goulvestre, C., Alexandre, J., Alves, A., Levy, E., Goldwasser, F., Panis, Y., Soubrane, O., Weill, B., and Batteux, F. (2005) Controlling tumor growth by modulating endogenous production of reactive oxygen species, *Cancer Res. 65*, 948-956.

7. Aronson, D. (2008) Hyperglycemia and the pathobiology of diabetic complications, *Adv. Cardiol. 45*, 1-16.

8. Ross, M. F., Kelso, G. F., Blaikie, F. H., James, A. M., Cocheme, H. M., Filipovska, A., Da Ros, T., Hurd, T. R., Smith, R. A., and Murphy, M. P. (2005) Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology, *Biochem. (Mosc.) 70*, 222-230.

9. Adlam, V. J., Harrison, J. C., Porteous, C. M., James, A. M., Smith, R. A., Murphy, M. P., and Sammut, I. A. (2005) Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury, *FASEB J. 19*, 1088-1095.

10. Smith, R. A., Porteous, C. M., Gane, A. M., and Murphy, M. P. (2003) Delivery of bioactive molecules to mitochondria in vivo, *PNAS USA 100*, 5407-5412.

11. Smith, R. A., Porteous, C. M., Coulter, C. V., and Murphy, M. P. (1999) Selective targeting of an antioxidant to mitochondria, *Eur. J. Biochem. 263*, 709-716.

12. Erster, S., Mihara, M., Kim, R. H., Petrenko, O., and Moll, U. M. (2004) In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation, *Mol. Cell Biol. 24*, 6728-6741.

13. Erster, S., and Moll, U. M. (2005) Stress-induced p53 runs a transcription-independent death program, *Biochem. Biophys. Res. Commun. 331*, 843-850.

14. Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis, *Science 303*, 1010-1014.

15. Chipuk, J. E., Maurer, U., Green, D. R., and Schuler, M. (2003) Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription, *Cancer Cell 4*, 371-381.

16. Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex, *Nat. Cell Biol. 6*, 443-450.

17. Pietsch, E. C., Leu, J. I., Frank, A., Dumont, P., George, D. L., and Murphy, M. E. (2007) The tetramerization domain of p53 is required for efficient BAK oligomerization, *Cancer Biol. Ther. 6*, 1576-1583.

18. Tomita, Y., Marchenko, N., Erster, S., Nemajerova, A., Dehner, A., Klein, C., Pan, H., Kessler, H., Pancoska, P., and Moll, U. M. (2006) WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization, *J. Biol. Chem. 281*, 8600-8606.

19. Kaufmann, T., Schlipf, S., Sanz, J., Neubert, K., Stein, R., and Borner, C. (2003) Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane, *J. Cell Biol. 160*, 53-64.

20. Pollack, M., and Leeuwenburgh, C. (2001) Apoptosis and aging: role of the mitochondria, *J. Gerontol. A Biol. Sci. Med. Sci. 56*, B475-482.

21. Lindsay, J., Esposti, M. D., and Gilmore, A. P. (2011) Bcl-2 proteins and mitochondria--specificity in membrane targeting for death, *Biochim. et Biophys. Acta. 1813*, 532-539.

22. Rehling, P., Brandner, K., and Pfanner, N. (2004) Mitochondrial import and the twin-pore translocase, *Nat. Rev. Mol. Cell Biol. 5*, 519-530.

23. Power, S. D., Lochrie, M. A., Patterson, T. E., and Poyton, R. O. (1984) The nuclear-coded subunits of yeast cytochrome c oxidase. II. The amino acid

sequence of subunit VIII and a model for its disposition in the inner mitochondrial membrane, *J. Biol. Chem. 259*, 6571-6574.

24. Fabrizi, G. M., Sadlock, J., Hirano, M., Mita, S., Koga, Y., Rizzuto, R., Zeviani, M., and Schon, E. A. (1992) Differential expression of genes specifying two isoforms of subunit VIa of human cytochrome c oxidase, *Gene 119*, 307-312.

25. Isaya, G., Fenton, W. A., Hendrick, J. P., Furtak, K., Kalousek, F., and Rosenberg, L. E. (1988) Mitochondrial import and processing of mutant human ornithine transcarbamylase precursors in cultured cells, *Mol. Cell Biol. 8*, 5150-5158.

26. Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) p53 has a direct apoptogenic role at the mitochondria, *Mol. Cell 11*, 577-590.

27. Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A., and Rotter, V. (1990) Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis, *Mol. Cell Biol. 10*, 6565-6577.

28. Kanwal, C., Mu, S., Kern, S. E., and Lim, C. S. (2004) Bidirectional on/off switch for controlled targeting of proteins to subcellular compartments, *J. Control. Release 98*, 379-393.

29. Kakar, M., Davis, J. R., Kern, S. E., and Lim, C. S. (2007) Optimizing the protein switch: altering nuclear import and export signals, and ligand binding domain, *J. Control. Release 120*, 220-232.

30. Wan, Y., Coxe, K. K., Thackray, V. G., Housley, P. R., and Nordeen, S. K. (2001) Separable features of the ligand-binding domain determine the differential subcellular localization and ligand-binding specificity of glucocorticoid receptor and progesterone receptor, *Mol. Endocrinol. 15*, 17-31.

31. Nalepa, G., Rolfe, M., and Harper, J. W. (2006) Drug discovery in the ubiquitin-proteasome system, *Nat. Rev. Drug Discov. 5*, 596-613.

32. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53, *Nature 387*, 296-299.

33. Chan, W. M., Mak, M. C., Fung, T. K., Lau, A., Siu, W. Y., and Poon, R. Y. (2006) Ubiquitination of p53 at multiple sites in the DNA-binding domain, *Mol. Cancer Res. 4*, 15-25.

34. Qiao, M., Chen, D., Hao, T., Zhao, X., Hu, H., and Ma, X. (2008) Injectable thermosensitive PLGA-PEG-PLGA triblock copolymers-based hydrogels as carriers for interleukin-2, *Pharmazie 63*, 27-30.

35. Chen, S., and Singh, J. (2008) Controlled release of growth hormone from thermosensitive triblock copolymer systems: In vitro and in vivo evaluation, *Int. J. Pharmaceu. 352*, 58-65.

36. Yu, L., Chang, G. T., Zhang, H., and Ding, J. D. (2008) Injectable block copolymer hydrogels for sustained release of a PEGylated drug, *Int. J. Pharmaceu. 348*, 95-106.

37. Choi, S., Baudys, M., and Kim, S. W. (2004) Control of blood glucose by novel GLP-1 delivery using biodegradable triblock copolymer of PLGA-PEG-PLGA in type 2 diabetic rats, *Pharmaceu. Res. 21*, 827-831.

38. Choi, S., and Kim, S. W. (2003) Controlled release of insulin from injectable biodegradable triblock copolymer depot in ZDF rats, *Pharmaceu. Res. 20*, 2008-2010.

39. McManus, M. J., Murphy, M. P., and Franklin, J. L. (2011) The Mitochondria-Targeted Antioxidant MitoQ Prevents Loss of Spatial Memory Retention and Early Neuropathology in a Transgenic Mouse Model of Alzheimer's Disease, *J. Neurosci. 31*, 15703-15715.

40. Mattarei, A., Biasutto, L., Marotta, E., De Marchi, U., Sassi, N., Garbisa, S., Zoratti, M., and Paradisi, C. (2008) A mitochondriotropic derivative of quercetin: a strategy to increase the effectiveness of polyphenols, *Chembiochem. 9*, 2633-2642.

41. Schuler, M., and Green, D. R. (2001) Mechanisms of p53-dependent apoptosis, *Biochem. Society Trans. 29*, 684-688.

42. Hagn, F., Klein, C., Demmer, O., Marchenko, N., Vaseva, A., Moll, U. M., and Kessler, H. (2010) BclxL changes conformation upon binding to wild-type but not mutant p53 DNA binding domain, *J. Biol. Chem. 285*, 3439-3450.

43. Xu, H., Tai, J., Ye, H., Kang, C. B., and Yoon, H. S. (2006) The N-terminal domain of tumor suppressor p53 is involved in the molecular interaction with the anti-apoptotic protein Bcl-Xl, *Biochem. Biophys. Res. Commun. 341*, 938-944.

44. Sayan, B. S., Sayan, A. E., Knight, R. A., Melino, G., and Cohen, G. M. (2006) p53 is cleaved by caspases generating fragments localizing to mitochondria, *J. Biol. Chem. 281*, 13566-13573.

45. Bharatham, N., Chi, S. W., and Yoon, H. S. (2011) Molecular Basis of Bcl-X(L)-p53 Interaction: Insights from Molecular Dynamics Simulations, *PLoS One 6*, e26014.

46. Kreiss, P., Cameron, B., Rangara, R., Mailhe, P., Aguerre-Charriol, O., Airiau, M., Scherman, D., Crouzet, J., and Pitard, B. (1999) Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency, *Nucleic Acids Res. 27*, 3792-3798.

47. Petros, A. M., Gunasekera, A., Xu, N., Olejniczak, E. T., and Fesik, S. W. (2004) Defining the p53 DNA-binding domain/Bcl-x(L)-binding interface using NMR, *FEBS Lett. 559*, 171-174.

48. Dubyak, G. R. (2011) Charge of the mito brigade. Focus on "Changes in mitochondrial surface charge mediate recruitment of signaling molecules during apoptosis", *Am. J. Physiol. Cell Physiol. 300*, C11-13.

49. Taylor, C. M., and Keating, A. E. (2005) Orientation and oligomerization specificity of the Bcr coiled-coil oligomerization domain, *Biochem. 44*, 16246-16256.

50. Moll, U. M., Riou, G., and Levine, A. J. (1992) Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion, *PNAS USA 89*, 7262-7266.

51. Kastan, M. B., and Zambetti, G. P. (2003) Parc-ing p53 in the cytoplasm, *Cell 112*, 1-2.

52. Dixon, A. S., Kakar, M., Schneider, K. M., Constance, J. E., Paullin, B. C., and Lim, C. S. (2009) Controlling subcellular localization to alter function: Sending oncogenic Bcr-Abl to the nucleus causes apoptosis, *J. Control. Release 140*, 245-249.

53. Rowley, J. D. (1973) Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining, *Nature 243*, 290-293.

54. Vigneri, P., and Wang, J. Y. (2001) Induction of apoptosis in chronic myelogenous leukemia cells through nuclear entrapment of BCR-ABL tyrosine kinase, *Nat. Med. 7*, 228-234.

55. Calabretta, B., and Perrotti, D. (2004) The biology of CML blast crisis, *Blood 103*, 4010-4022.

56. Oldham, R., and Dillman, R. (2004) Gold standard or wrong standard?, *Cancer Biother. Radiopharm. 19*, 271-272.

57. Peggs, K. (2004) Imatinib mesylate--gold standards and silver linings, *Clin. Exp. Med. 4*, 1-9.

58. Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J., and Lydon, N. B. (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells, *Nat. Med. 2*, 561-566.

59. Baccarani, M., Saglio, G., Goldman, J., Hochhaus, A., Simonsson, B., Appelbaum, F., Apperley, J., Cervantes, F., Cortes, J., Deininger, M., Gratwohl, A., Guilhot, F., Horowitz, M., Hughes, T., Kantarjian, H., Larson, R., Niederwieser, D., Silver, R., and Hehlmann, R. (2006) Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet, *Blood 108*, 1809-1820.

60. Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N., and Sawyers, C. L. (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification, *Science 293*, 876-880.

61. Shah, N. P., and Sawyers, C. L. (2003) Mechanisms of resistance to STI571 in Philadelphia chromosome-associated leukemias, *Oncogene 22*, 7389-7395.

62. Dixon, A. S., Pendley, S. S., Bruno, B. J., Woessner, D. W., Shimpi, A. A., Cheatham, T. E., 3rd, and Lim, C. S. (2011) Disruption of Bcr-Abl coiled coil oligomerization by design, *J. Biol. Chem. 286*, 27751-27760.

63. Cotrim, A. P., and Baum, B. J. (2008) Gene therapy: some history, applications, problems, and prospects, *Toxicol. Pathol. 36*, 97-103.

64. Merritt, J. A., Roth, J. A., and Logothetis, C. J. (2001) Clinical evaluation of adenoviral-mediated p53 gene transfer: review of INGN 201 studies, *Semin. Oncol. 28*, 105-114.

65. Roth, J. A., Grammer, S. F., Swisher, S. G., Komaki, R., Nemunaitis, J., Merritt, J., Fujiwara, T., and Meyn, R. E., Jr. (2001) Gene therapy approaches for the management of non-small cell lung cancer, *Semin. Oncol. 28*, 50-56.

66. Gerdes, C. A., Castro, M. G., and Lowenstein, P. R. (2000) Strong promoters are the key to highly efficient, noninflammatory and noncytotoxic adenoviral-mediated transgene delivery into the brain in vivo, *Mol. Ther. 2*, 330-338.

67. Khuri, F. R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I. F., Romel, L., Gore, M., Ironside, J., MacDougall, R. H., Heise, C., Randlev, B., Gillenwater, A. M., Bruso, P., Kaye, S. B., Hong, W. K., and Kirn, D. H. (2000) a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer, *Nat. Med. 6*, 879-885.

68. Thomas, C. E., Ehrhardt, A., and Kay, M. A. (2003) Progress and problems with the use of viral vectors for gene therapy, *Nat. Rev. Genet. 4*, 346-358.

69. Bao, R., Connolly, D. C., Murphy, M., Green, J., Weinstein, J. K., Pisarcik, D. A., and Hamilton, T. C. (2002) Activation of cancer-specific gene expression by the survivin promoter, *J. Natl. Cancer Inst. 94*, 522-528.

70. Blask, D. E., Dauchy, R. T., Sauer, L. A., Krause, J. A., and Brainard, G. C. (2003) Growth and fatty acid metabolism of human breast cancer (MCF-7) xenografts in nude rats: impact of constant light-induced nocturnal melatonin suppression, *Breast Cancer Res. Treat. 79*, 313-320.

71. Bao, R., Connolly, D. C., Murphy, M., Green, J., Weinstein, J. K., Pisarcik, D. A., and Hamilton, T. C. (2002) Activation of cancer-specific gene expression by the survivin promoter, *J. Natl. Cancer Inst. 94*, 522-528.

72. Zhang, J. F., Hu, C., Geng, Y., Selm, J., Klein, S. B., Orazi, A., and Taylor, M. W. (1996) Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy, *PNAS USA 93*, 4513-4518.

73. Lang, F. F., Bruner, J. M., Fuller, G. N., Aldape, K., Prados, M. D., Chang, S., Berger, M. S., McDermott, M. W., Kunwar, S. M., Junck, L. R., Chandler, W., Zwiebel, J. A., Kaplan, R. S., and Yung, W. K. (2003) Phase I trial of adenovirus-mediated p53 gene therapy for recurrent glioma: biological and clinical results, *J. Clin. Oncol. 21*, 2508-2518.

74. Blask, D. E., Sauer, L. A., Dauchy, R. T., Holowachuk, E. W., Ruhoff, M. S., and Kopff, H. S. (1999) Melatonin inhibition of cancer growth in vivo involves suppression of tumor fatty acid metabolism via melatonin receptor-mediated signal transduction events, *Cancer Res. 59*, 4693-4701.

75. Dauchy, R. T., Sauer, L. A., Blask, D. E., and Vaughan, G. M. (1997) Light contamination during the dark phase in "photoperiodically controlled" animal rooms: effect on tumor growth and metabolism in rats, *Lab Anim. Sci. 47*, 511-518.

76. Dauchy, R. T., Blask, D. E., Sauer, L. A., Brainard, G. C., and Krause, J. A. (1999) Dim light during darkness stimulates tumor progression by enhancing tumor fatty acid uptake and metabolism, *Cancer Lett. 144*, 131-136.

77. Janat-Amsbury, M. M., Yockman, J. W., Lee, M., Kern, S., Furgeson, D. Y., Bikram, M., and Kim, S. W. (2004) Combination of local, nonviral IL12 gene therapy and systemic paclitaxel treatment in a metastatic breast cancer model, *Mol. Ther. 9*, 829-836.

78. Janat-Amsbury, M. M., Yockman, J. W., Lee, M., Kern, S., Furgeson, D. Y., Bikram, M., and Kim, S. W. (2005) Local, non-viral IL-12 gene therapy using a

water soluble lipopolymer as carrier system combined with systemic paclitaxel for cancer treatment, *J. Control. Release 101*, 273-285.

79. Furgeson, D. Y., Yockman, J. W., Janat, M. M., and Kim, S. W. (2004) Tumor efficacy and biodistribution of linear polyethylenimine-cholesterol/DNA complexes, *Mol. Ther. 9*, 837-845.

80. Lee, E. S., Gao, Z., Kim, D., Park, K., Kwon, I. C., and Bae, Y. H. (2008) Super pH-sensitive multifunctional polymeric micelle for tumor pH(e) specific TAT exposure and multidrug resistance, *J. Control. Release 129*, 228-236.

81. Mohajer, G., Lee, E. S., and Bae, Y. H. (2007) Enhanced intercellular retention activity of novel pH-sensitive polymeric micelles in wild and multidrug resistant MCF-7 cells, *Pharm. Res. 24*, 1618-1627.

82. Lee, E. S., Na, K., and Bae, Y. H. (2005) Doxorubicin loaded pH-sensitive polymeric micelles for reversal of resistant MCF-7 tumor, *J. Control. Release 103*, 405-418.

83. Gangadharan, C., Thoh, M., and Manna, S. K. (2009) Inhibition of constitutive activity of nuclear transcription factor kappaB sensitizes doxorubicin-resistant cells to apoptosis, *J. Cell Biochem 107*, 203-213.

84. Draize, J. H., and Kelley, E. A. (1959) The urinary excretion of boric acid preparations following oral administration and topical applications to intact and damaged skin of rabbits, *Toxicol. 1*, 267-276.

85. Kiernan, J. A. (1981) *Histological and Histochemical Methods: Theory and Practice*, 1st ed., Pergamon Press, Elmsford, NY.

86. Edinger, A. L., and Thompson, C. B. (2004) Death by design: apoptosis, necrosis and autophagy, *Curr. Opin. Cell Biol. 16*, 663-669.

87. Festjens, N., Vanden Berghe, T., and Vandenabeele, P. (2006) Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response, *Biochim. Biophys. Acta. 1757*, 1371-1387.

88. Haziot, A., Tsuberi, B. Z., and Goyert, S. M. (1993) Neutrophil CD14: biochemical properties and role in the secretion of tumor necrosis factor-alpha in response to lipopolysaccharide, *J. Immunol. 150*, 5556-5565.

89. Li, S., Wu, S. P., Whitmore, M., Loeffert, E. J., Wang, L., Watkins, S. C., Pitt, B. R., and Huang, L. (1999) Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors, *Am. J. Physiol. 276*, L796-804.